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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 ≥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 (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; $P = 0.66$, $\chi^2$ 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%; $P = 0.28$, $\chi^2$ 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, $P = 0.5$), 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%, $P < 0.01$; $\chi^2$ 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.

**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× 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 ≥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 ($n$ = 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.

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.

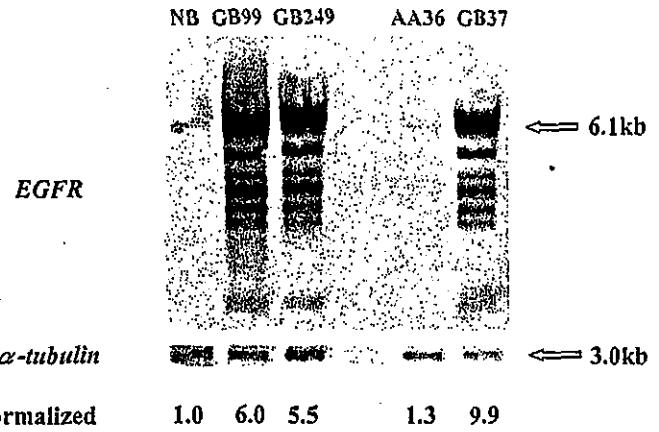


Fig. 1. *EGFR* gene amplification detected by Southern blot analysis in GBM tumor samples. Comparison of the signal intensity of a 6.1-kb *Eco*RI fragment of an *EGFR* gene and a 3-kb fragment of an *α-tubulin* gene in tumor samples (GB99, GB249, AA36, and GB37) and normal brain tissue samples (NB).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

***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*

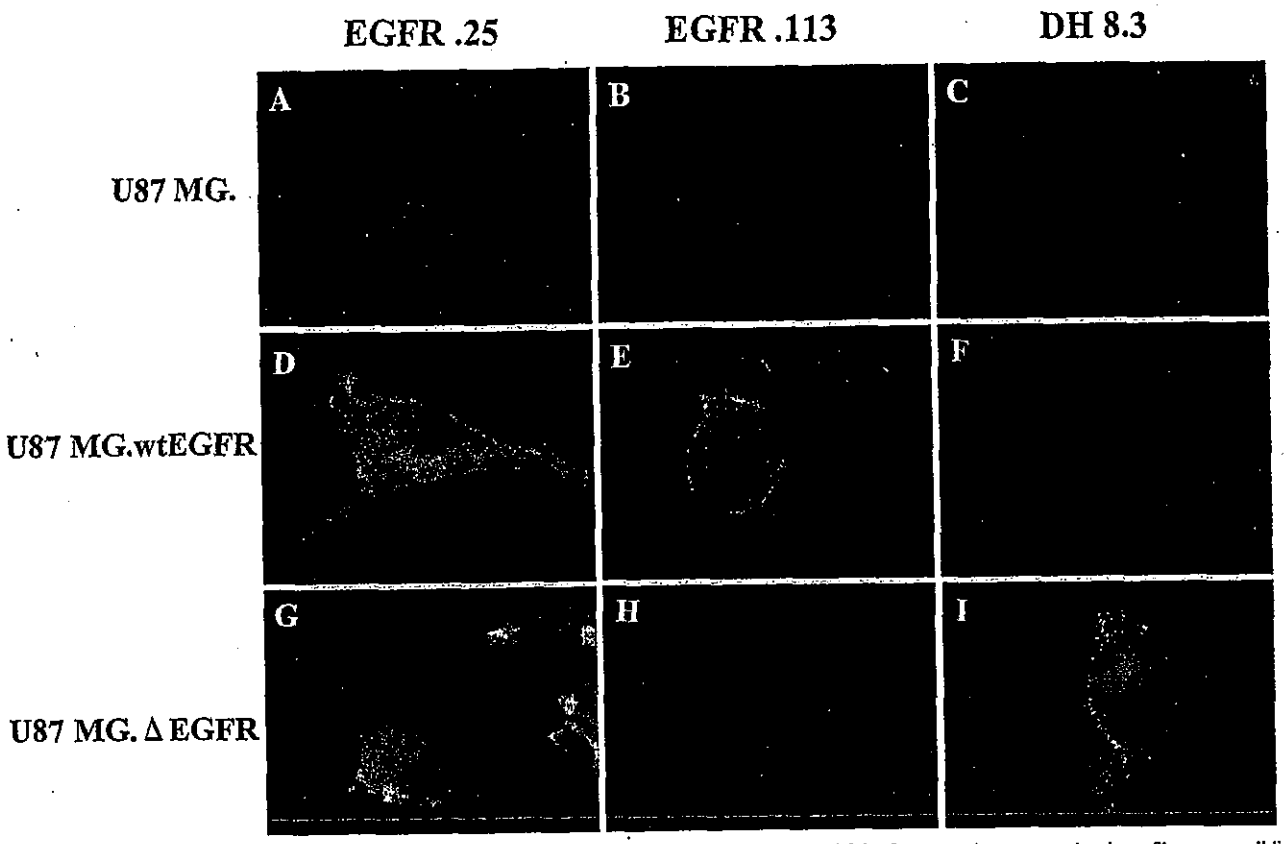


Fig. 2. Immunoreactivity with EGFRwt and EGFRvIII of monoclonal antibodies EGFR.25, EGFR.113, and DH8.3 by fluorescent immunocytochemistry. Shown are cell lines U 87MG. parental cells (*A–C*), U87 MG.wtEGFR cells (*D–F*), and U87 MG.ΔEGFR cells (*G–I*). The cells were treated with the following monoclonal 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 ($P = 0.038$,

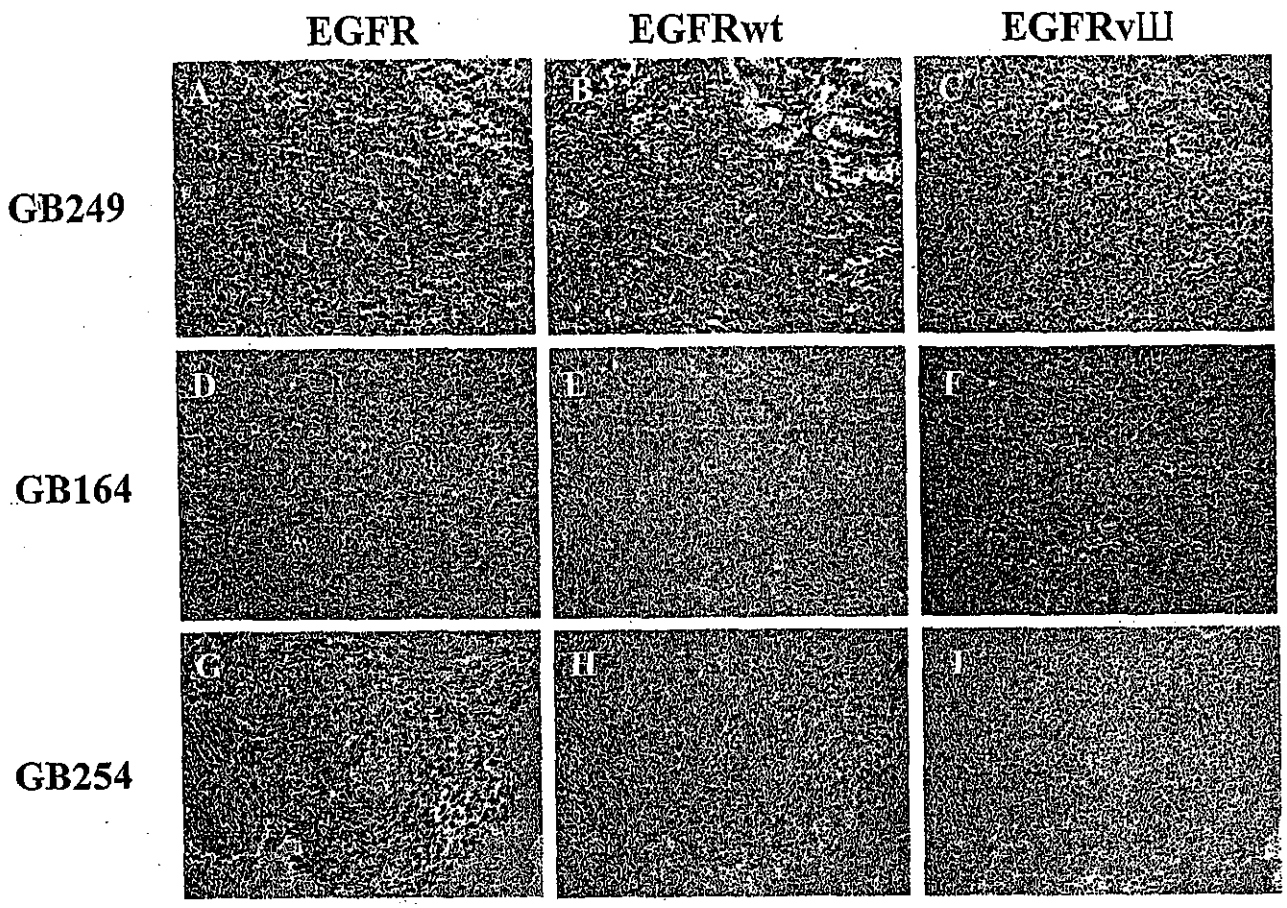


Fig. 3. Expression patterns of each EGFR in three cases with *EGFR* amplification. Shown are three illustrative cases with *EGFR* amplification. The expression of EGFR, EGFRwt, and EGFRvIII is shown in the *left, center,* and *right panels,* respectively. GB249 (*A–C*), the same case as shown in Fig. 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

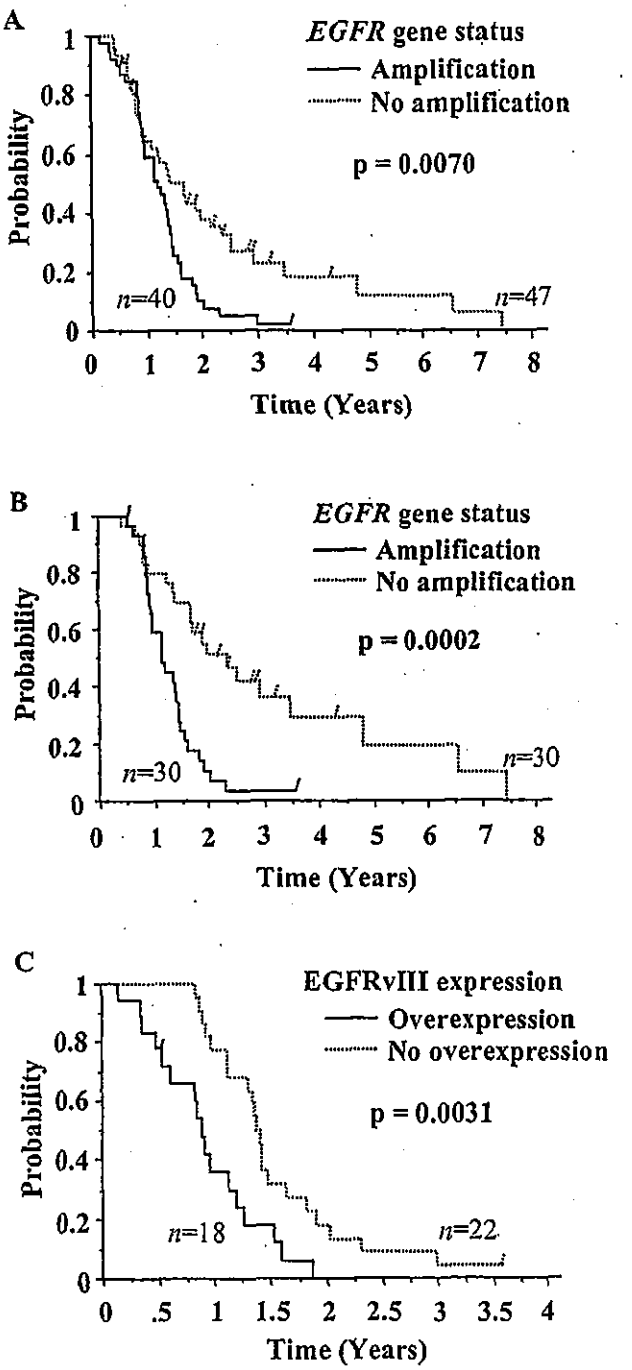


Fig. 4. OS according to *EGFR* gene status and EGFRvIII expression in 87 patients with GBM. Shown are Kaplan-Meier survival curves of OS for all 87 patients, irrespective of the presence or absence of amplification (*A*), 60 patients < 60 years, irrespective of the presence or absence of amplification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

| Age group | Median OS, yrs (no. of cases) | | | Multivariate (Amp. *vs.* no amp.) | |
|---|---|---|---|---|---|
| | 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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# Expression of the Oligodendroglial Lineage-Associated Markers Olig1 and Olig2 in Different Types of Human Gliomas

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**Abstract.** Because a specific group of oligodendrogliomas is susceptible to adjuvant therapy, it is important to elucidate the biological characteristics of these tumors. In situ hybridization analyses have revealed that *Olig* genes are expressed in oligodendroglial lineage cells and are highly expressed in oligodendrogliomas. To clarify whether OLIG is a tumor-specific marker for oligodendrogliomas, we have investigated the expression of *Olig* transcripts by semiquantitative RT-PCR assay and OLIG2 protein with a new antibody in a variety of glial tumors. The semiquantitative RT-PCR revealed that high levels of expression of *Olig1* and *Olig2* mRNAs were present in anaplastic oligodendrogliomas and anaplastic astrocytomas, while expression of these mRNAs in grade IV glioblastomas was lower than in grade II and grade III gliomas ($p < 0.01$). Immunohistochemical analyses demonstrated that the mean immunopositive proportion of OLIG2 was 82% in anaplastic oligodendrogliomas but only 34% in anaplastic astrocytomas. Therefore, although OLIG2 expression was detected in a range of gliomas not specific for oligodendrogliomas, the expression level in anaplastic oligodendrogliomas was more uniform and intense than that in other glial tumors. In conclusion, combining *Olig* mRNA expression and immunohistochemistry of OLIG2 enables oligodendrogliomas to be distinguished from glioblastomas and other astrocytic glial tumors.

**Key Words:** Anti-OLIG2 antibody; Auxiliary diagnostic tool; Human gliomas; Immunohistochemistry; *Olig1*; *Olig2*; Quantitative RT-PCR.

## INTRODUCTION

Classifications of glial tumors have been based upon the system of Bailey and Cushing, who classified glial neoplasms on the basis of morphological similarities of tumor cells to non-neoplastic cells (1, 2). Although rapid progress in molecular and developmental biology has disclosed some aspects of glial tumors, their histological features and clinical behavior have not been fully explained by the known genetic background alone. Information on the genetic abnormalities of glial tumors should enable the glial tumors to be reclassified according to their genetic characteristics. This will allow the patient prognosis to be predicted and the sensitivity of the glial tumors to specific therapies to be determined.

Extensive attention has been paid to oligodendroglial tumors because a good prognosis for the patient is correlated to specific genetic abnormalities and chemotherapeutic sensitivity. The loss of heterozygosity (LOH) of 1p and 19q was recognized in about 80% to 90% of grade II oligodendrogliomas, 50% to 70% of grade III anaplastic oligodendrogliomas, and 50% of grade II oligoastrocytomas (3–6), and has been shown to be a marker indicative of their susceptibility to chemotherapy and/or irradiation (6–9). Combined loss of 1p and 19q is, however, a statistically significant positive predictor of overall survival only in oligodendroglioma patients and not in grade IV GBM or in mixed oligoastrocytoma patients (3).

In addition, tumor cells exhibiting a perinuclear halo, which is a typical histologic diagnostic criterion of oligodendrogliomas, have been observed in various other types of tumors, which suggests that histopathologic features alone might be insufficient for the diagnosis of oligodendroglial tumors. Thus, the genetic characterization of oligodendrogliomas would be helpful to obtain possible diagnostic markers for the tumor.

The *Olig* genes constitute a recently identified family of basic helix-loop-helix (bHLH) transcription factors that were found to be specifically expressed in cells of the oligodendrocyte lineage (10, 11). *OLIG* expression induces differentiation of the neural progenitors to form oligodendrocytes. *OLIGs* are also expressed in oligodendrocytes themselves and in the putative immature progenitor cells of the adult central nervous system (CNS) (12–15). Although high expression levels of *Olig* genes have already been demonstrated in oligodendroglial tumors (16, 17), the relationship between the OLIG protein expression and the histological characteristics of various glial tumors, with regard to tumor type and WHO grade, has not been elucidated. To clarify the significance of OLIG expression in oligodendroglial tumors we assessed the expression of OLIG in various glial tumors examining

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the expression levels of *Olig* mRNAs by the semiquantitative RT-PCR and OLIG2 protein by immunohistochemical assay with a newly generated specific antibody.

## MATERIALS AND METHODS

### Primary Glial Tumor Samples

Primary brain tumor samples were obtained from patients treated at the Department of Neurosurgery, Hokkaido University School of Medicine between 1995 and 2002. Informed consent was obtained from all patients in this study for subsequent use of their resected tissues. The collection of tumor samples was preserved by formalin fixation and frozen preservation, respectively. For the histologic examination, hematoxylin and eosin (H&E) staining was performed from formalin-fixed, paraffin-embedded tissues. The samples were taken from the same tumor areas and were used for frozen sectioning. We made H&E-stained preparations to confirm that the frozen tissue actually contained tumor cells. These H&E sections were reviewed and classified according to the WHO grading system independently by 2 neuropathologists (KN and AO). The diagnostic discrepancies were resolved by mutual agreement after sufficient discussion and a few cases were excluded from the study because of the lack of agreement with the diagnosis. The examined tumors comprised 10 pilocytic astrocytomas (PA) WHO grade I, 5 low-grade diffuse astrocytomas (AS) WHO grade II, 1 oligodendrogliomas (OL) and 6 oligoastrocytomas (OA) WHO grade II, 9 anaplastic astrocytomas (AA) WHO grade III, 6 anaplastic oligodendrogliomas (AO) WHO grade III, 2 anaplastic ependymomas (AE) WHO grade III, and 9 glioblastomas (GBM) WHO grade IV. All 48 cases were examined by semiquantitative RT-PCR and immunohistochemical staining with the specific antibody for OLIG2.

### Cell Culture Conditions

Human glioblastoma cell lines A172, T98G, U87MG, U251MG, and human adrenal carcinoma line SW-13 were maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) from Sigma (St. Louis, MO) at 5% $CO_2$.

### RNA Preparation and Semiquantitative RT-PCR

Before extraction of RNA from frozen samples, 10-μm samples were cut by cryostat, mounted on glass slides, stained with H&E and examined. Total RNAs were extracted from all 48 frozen tumor samples using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of DNase-treated total RNAs in a volume of 20 μl catalyzed by SuperScript II RNase H⁻ Reverse Transcriptase using Oligo $(dT)_{12-18}$ primers (Invitrogen Corp., Carlsbad, CA). Primers for *Olig1*, *Olig2* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*Olig1*: forward primer, 5'-CTA AAA TAG GTA ACC AGG CGT CTC A-3'; reverse primer, 5'-CCC GGT ACT CCT GCG TGT T-3'; *Olig2*: forward primer, 5'-TGC GCA AGC TTT CCA AGA T-3'; reverse primer, 5'-CAG CGA GTT GGT GAG CAT GA-3'; GAPDH: forward primer, 5'-GAA GGT GAA GGT CGG AGT C-3'; reverse primer; 5'-GAA GAT GGT GAT GGG ATT TC-3') were synthesized. To examine the levels of mRNA expression of *Olig1* and *Olig2*, PCR amplification and analyses were achieved using Gene Amp 5700 and software version 1.3 (Applied Biosystems, Foster City, CA). The PCR solution (50 μl) was composed of 0.2 μl of cDNA solution, 10 pmol of the forward and reverse primers, and 25 μl of 2× SYBR Green PCR Master Mix. PCR was carried out after incubation at 50°C for 2 min, denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 61°C for 1 min. Experiments were independently performed in duplicate. A standard concentration sample was prepared by the admixture of 10 randomly selected glial tumor samples. Adult brain cDNA (Invitrogen) was used as a control. For each experimental sample, the amounts of *Olig1*, *Olig2*, and GAPDH mRNAs were quantified from standard curves. GAPDH transcripts were monitored as an internal control to qualify the transcripts of the genes in each sample. The normalized amounts of *Olig1* and *Olig2* mRNAs were determined by dividing the amounts of *Olig1* and *Olig2* mRNAs by the amount of GAPDH mRNA for each sample.

### Generation and Purification of Polyclonal Antibodies to OLIG2

The antibody specific to OLIG2 was produced using a synthesized peptide corresponding to amino acid residues 64–77 of OLIG2. The peptide was synthesized with an additional cysteine at its amino terminus to facilitate the coupling to the carrier protein, keyhole limpet hemocyanin (KLH). Rabbits were immunized with the peptide-KLH conjugate, and one of the antibodies named OG2 was purified by affinity chromatography with the synthetic peptide (Sigma Genosis Japan, Ishikari, Japan) and used for the further studies.

### Immunoblotting

To characterize the antibody (OG2) against OLIG2, 2 glial tumor samples that expressed *Olig2* mRNA were used as positive controls and 5 cell lines plus a metastatic brain tumor sample without any expression of *Olig2* mRNA (data not shown) were used as negative controls for immunoblotting.

Cultured cells and frozen tissues were lysed with a buffer composed of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 mM sodium orthovanadate ($Na_3VO_4$) for 10 min on ice, and were centrifuged at 15,000 rpm for 10 min at 4°C. After measurement of the protein concentrations, supernatants were subjected to SDS-PAGE and the separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon, Millipore, Billerica, MA) as described previously (18). The membranes were blocked for 2 h at room temperature in 5% nonfat dry milk in TBS-T buffer (Tris-buffered saline containing 0.1% Tween-20) and then incubated with the anti-OLIG2 antibody OG2 (1:1,000) diluted in TBS-T buffer at room temperature for 2 h. After washing 3 times, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulins (1:5,000) (Biosource, Camarillo, CA). The immunopositive signals were detected using ECL Plus Western blotting detection reagents (Amersham Biosciences, Uppsala, Sweden) and

quantified using a lumino image analyzer (LAS1000: Fuji Film, Tokyo, Japan).

### Immunohistochemistry

To examine the localization and expression of the OLIG2 protein, all 48 cases including 2 cases of AE that did not express *Olig2* mRNA were immunostained with a rabbit anti-human OLIG2 polyclonal antibody OG2 (diluted to 1:200). Formalin-fixed, paraffin-embedded tissue blocks were cut at 4-μm thickness, transferred onto glass slides, deparaffinized with xylene, and treated with 0.1 mM EDTA buffer (pH 8.0) in a pressure cooker for 2.5 min for antigen retrieval before immunostaining. The slides were stained using the DAKO EnVision + System (Dako Cytomation, Kyoto, Japan) and the Ventana automated immunostainer (Ventana, Tucson, AZ).

The immunopositive index of *OLIG2* was represented as the percent of positive cells per 1,000 tumor cells. For the calculation of immunopositive index of OLIG2, an area with typical histopathologic features as agreed by 2 pathologists was selected. In the case of PA, which was composed of 2 different components, including compact fibrous and loose myxoid regions, the positive index was calculated separately.

### Statistical Analysis

Differences in the numerical data between different tumor entities and WHO grades were evaluated using the Mann-Whitney *U*-test. Probability (p) values were 2-tailed. A p value of 0.05 or less was considered statistically significant. Two cases of anaplastic ependymomas were excluded from the statistical analysis because of the small number of cases examined.

## RESULTS

### *Olig1* and *Olig2* mRNA Expression in Primary Glial Tumors

*Olig1* and *Olig2* mRNAs were detected not only in OL/OA and AO, but also in various glial tumors. The mean expression levels of *Olig1* and *Olig2 mRNAs* in the tumor samples are summarized according to the categories of glial tumors (Fig. 1A). The *Olig1* mRNA expression level was relatively high in OL/OA, AA, and AO, and in particular, higher expression of *Olig1* was observed in OL/OA compared to AS and PA ($p < 0.05$). In GBM, expression of *Olig1* was least in the astrocytomas including those of grades I, II, and III (*Olig1*: $p < 0.05$) and in the oligodendrogliomas of grades II and III (*Olig1*: $p < 0.05$). The expression of *Olig2* mRNA was similar to that of *Olig1* in being relatively high in AA and AO among the glial tumors, but not so prominent in OL/OA. However, a low level of expression was also noted in the PA, AS, and GBM. Similarly in GBM, expression was lower than that shown by the astrocytomas (*Olig2*: $p < 0.01$) and oligodendrogliomas (*Olig2*: $p < 0.01$). When we compared the expression levels of both genes between astrocytomas of grades II and III and oligodendrogliomas of grades II and III there was no significant difference.

We also summarized the expression levels of *Olig1* and *Olig2* according to the WHO classification of gliomas (Fig. 1B). The expression levels of both *Olig1* and *Olig2* increased incrementally from grade I to III, but grade IV GBM was the lowest among the glial tumors and the statistical significance was evident compared to grade III (*Olig1*: $p < 0.01$, *Olig2*: $p < 0.01$). In 2 cases of AE, neither *Olig1* nor *Olig2* mRNA was detected.

### Characterization of the Anti-OLIG2 Antibody (OG2) by Immunoblotting

Immunoblot analyses using an anti-OLIG2 antibody, OG2, demonstrated an intense band of OLIG2 around 40 kDa in cases of anaplastic astrocytoma (#885) and anaplastic oligodendroglioma (#1379) (Fig. 2). In contrast, the signal of OLIG2 was not detectable in a metastatic brain tumor case (#1175) or in U87MG, U251MG, T98G, A172, and SW13 cells (Fig. 2).

### Localization of OLIG2 Protein in Glial Tumors

All 48 glial tumor samples were immunostained with the anti-OLIG2 antibody OG2. The immunopositive proportion of OLIG2 was represented by the mean fraction of OLIG2-positive tumor cells. The specificity was evaluated by surveying nuclei of vascular endothelial cells and a few intermingled reactive astrocytes, which did not take the brown label. OLIG2 protein was detected in the nuclei of glial tumor cells that are known to express *Olig2* mRNA. In AO, which expressed the highest mRNA (Fig. 1A), nuclear staining of OLIG2 was diffuse and intense (Fig. 3B). In 4 of 6 AO cases, the proportion of immunopositive cells was more than 90%, with an average of 82% (Table 3). In contrast, nuclear staining of AA was not diffuse, and many blue-stained nuclei (hematoxylin) were observed (Fig. 3D) and regarded as OLIG2-negative. The OLIG2 immunopositivity in AA varied between cases with a range from 4% to 66% and an average of 34% (Table 3). In AS, some nuclei of astrocytomas were also positively stained, but the intensity was weaker compared to AO. The proportion of positive nuclei was relatively high in protoplasmic astrocytomas, and quite low in both fibrillary astrocytomas and gemistocytic astrocytomas, while in grade II OL/OA tumors positive proportion was greater than 50% (Table 2; Fig. 4B).

In PA, which consists of a biphasic pattern, OLIG2-positive nuclei were found in the pilocytic cells of the loose and myxoid areas, while few positive nuclei were observed in the compact and fibrillary areas (Table 1; Fig. 4D). In GBM, OLIG2-positive nuclei were rarely observed (Table 1; Fig. 4F), consistent with the expression of mRNA. In 2 cases of AE, no OLIG2 expression was observed.

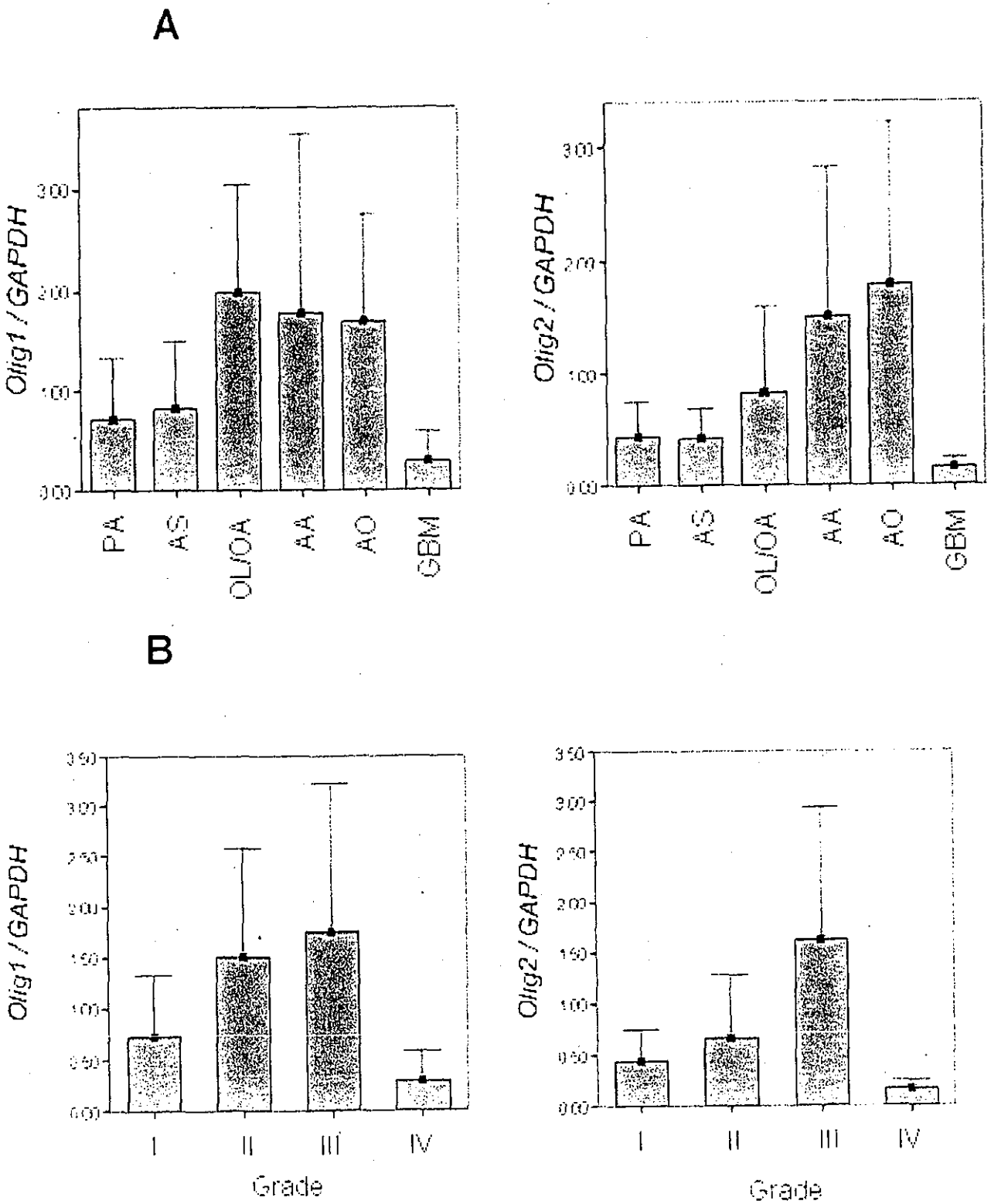


**Fig. 1.** A: The mean expression levels of *Olig1* and *Olig2* mRNAs in various glial tumors. The *Olig1* mRNA expression level was relatively high in OL/OA, AA, and AO. Higher expression of *Olig1* mRNA is observed in OL/OA compared to PA and AS ($p < 0.05$) The expression of *Olig2* mRNA was similar to that of *Olig1* in being relatively high in AA and AO, but not as prominent in OL/OA. B: The mean expression level of *Olig1* and *Olig2* mRNAs in various WHO grade tumors. The expression levels of both *Olig1* and *Olig2* increased incrementally from grade I to III, but grade IV GBM was the lowest among the glial tumors and the statistical significance was evident compared to grade III (*Olig1*: $p < 0.01$, *Olig2*: $p < 0.01$). Error bars represent +10% SE.

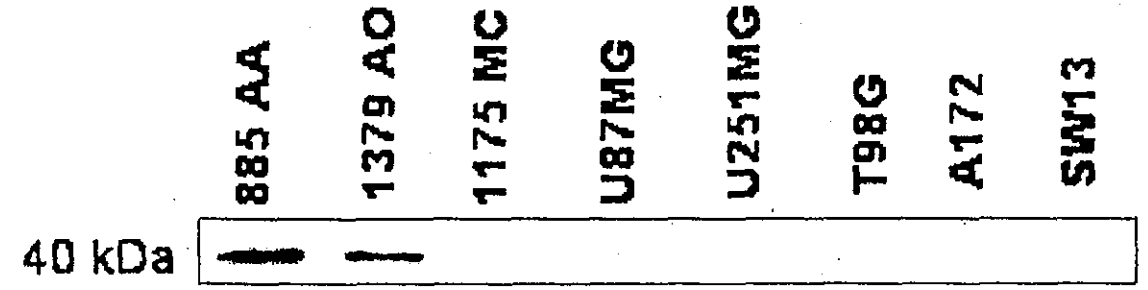


Fig. 2. Immunoblot analysis using a newly generated polyclonal antibody against OLIG2 of tissue samples and cell lines. The intense band of OLIG2 at 40 kDa was exclusively detected in AA and AO, but not in MC, U87MG, U251MG, T98G, A172, or SW13 cell line. 885 AA: Sample No 26. 1379 AO: Sample No 37, 1175 MC: metastatic cancer.

## DISCUSSION

*Olig* transcripts and OLIG2 protein expression were investigated in a range of glial tumors to clarify the biological nature of oligodendrogliomas. Marie et al examined the expression of *Olig2* in 12 cases of oligodendrogliomas and oligoastrocytomas and 4 cases of glioblastomas by in situ hybridization, which showed high expression levels of *Olig2* in all cases of oligodendrogliomas and oligoastrocytomas, but none in glioblastomas (16). Lu et al also observed high expression levels of *Olig1* and *Olig2* transcripts in 6 cases of oligodendrogliomas and weak expression in 1 of 9 cases of astrocytomas (including grades I, II, and III), and in 8 cases of glioblastomas (17). Supporting the findings of Marie et al and Lu et al (16, 17), *Olig* transcripts were highly expressed in oligodendrogliomas in our study. However, upregulation of *Olig* transcripts by RT-PCR assay was also detected in most of the glial tumors including astrocytomas, except for the majority of GBM.

It is important to visualize OLIG expression in histologic specimens to confirm that the tumor cells actually express this protein. Therefore, all 48 specimens were immunohistochemically examined using a new specific antibody against OLIG2. As indicated, OLIG2 was diffusely and uniformly stained in most nuclei of AO, accounting for more than 90% of immunopositivity in 4 of 6 cases. In a fifth case of AO, the immunopositive proportion could be lower than the actual staining because these tumor cells infiltrated into brain parenchyma too diffusely to recognize each positive cell. OLIG2 protein tends to be intensely and diffusely expressed in AO, however, more specimens should be examined to confirm these results. In OL/OA cases, approximately half of the tumor cells expressed OLIG2 in all samples regardless of histologic features, that is, oligo- or astro-components.

*Olig* expression was also detected in PA and other astrocytic glial tumors; however, the distribution of OLIG2 was inconsistent in these specimens. In PA, which is composed of fibrillary and myxoid areas, interestingly, OLIG2 was preferentially expressed in neoplastic cells in the myxoid area with a little expression in the fibrillary area. PA tumors express glial fibrillary acidic protein (GFAP) and belong to astrocytic glial tumors, but they seem to be a distinct entity because of the specific pathological characters, including biphasic pattern, specific incidence topography, and relatively good prognosis. The reason of OLIG2 in cells of myxoid areas of PA remains to be further examined. In AS, the immunopositive proportion was high in protoplasmic astrocytomas, but low in both fibrillary and gemistocytic astrocytomas. Although the number of AS cases were fewer, it is interesting that the expression pattern of OLIG2 was different according to the morphologic features. In particular, the OLIG2 was intensely expressed in protoplasmic astrocytomas with few flaccid processes, suggesting that the expression of OLIG2 might be related to the morphology of glia.

The mean immunopositive proportion was approximately one third in AA and no association between histologic appearances and OLIG2 positivity was observed. As high-grade glial tumors, including GBM, are genetically unstable and neoplastic cells of these tumors express a wide range of marker genes for different neural lineages (19–21), upregulation or inhibition of OLIG2 expression might have been occurring randomly in these tumors.

Thus, *Olig 2* mRNA and OLIG2 protein were expressed in various glial tumors, suggesting that OLIG2 seemed not to be a specific immunohistochemical marker of oligodendroglial tumors. However, it was shown that some specific immunostaining tendency of OLIG2 was recognized by categories of glial tumors. For instance, OLIG2 was uniformly distributed in AO and more intensely stained in protoplasmic type than in other astrocytomas and in myxoid area of PA and was very weakly expressed in GBM. It has been recognized that the histological diagnosis of oligodendrogliomas, especially differential diagnosis between AO and GBM is sometimes difficult. As the clinical course and the therapeutic strategy were quite different in these tumors, correct pathological diagnosis is very important. In addition, oligodendroglial tumors have been occasionally misdiagnosed

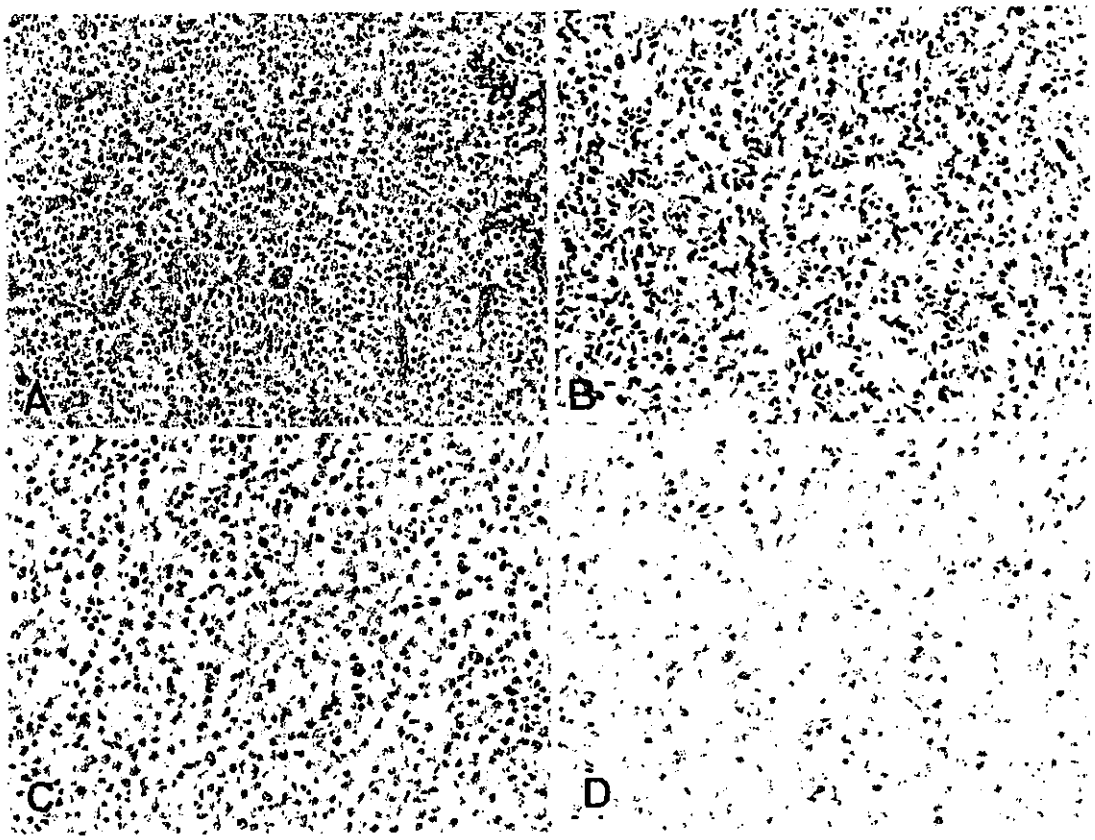


**Fig. 3.** Immunohistochemistry of OLIG2 in glial tumor specimens. H&E-stained sections of anaplastic oligodendroglioma (No 37) (**A**); anaplastic astrocytoma (No 26) (**C**). An exceptionally high positive proportion of OLIG2 is observed in anaplastic oligodendroglioma (**B**), moderate positivity in anaplastic astrocytoma (**D**).

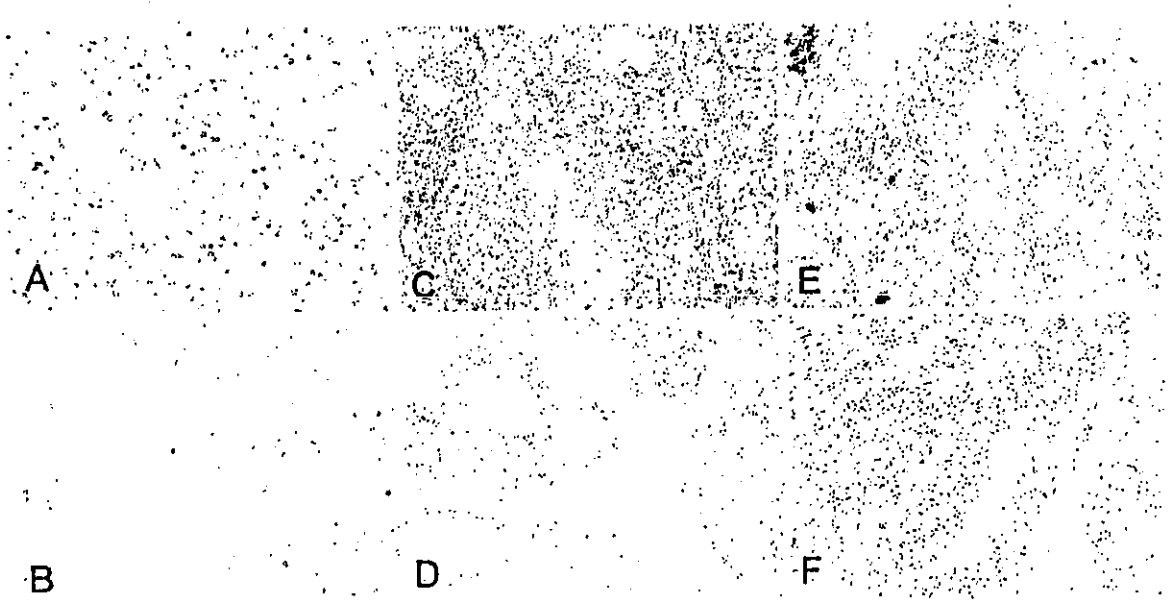


**Fig. 4.** Immunohistochemistry of OLIG2 in various glial tumor specimens. H&E-stained sections of oligoastrocytoma (No 21) (**A**); pilocytic astrocytoma (No 8) (**C**); and glioblastoma (No 44) (**E**). OLIG2 is observed in diffuse but weak positivity in oligoastrocytoma (**B**), and rare positive cells in glioblastoma (**F**). In pilocytic astrocytoma, OLIG2 was preferentially observed in the tumor cells at the myxoid area compared to the compact area (**D**).

TABLE 1
Immunohistochemistry of OLIG2 in PA

| Sample no. | Diag | Age | Location of tumor | Compact area (%) | Myxoid area (%) |
|---|---|---|---|---|---|
| 1 | PA | 1 | chiasm | — | 70 |
| 2 | PA | 1 | chiasm | — | 50 |
| 3 | PA | 7 | temporal | <1 | — |
| 4 | PA | 3 | cerebellum | 5 | 95 |
| 5 | PA | 3 | chiasm | 5 | 60 |
| 6 | PA | 7 | chiasm | 10 | 70 |
| 7 | PA | 7 | chiasm | 20 | 95 |
| 8 | PA | 5 | cerebellum | 30 | 90 |
| 9 | PA | 48 | frontal | <1 | — |
| 10 | PA | 24 | thalamus | 8 | 40 |

Diag: pathological diagnosis, PA: pilocytic astrocytoma, —: not contained.

TABLE 2
Immunohistochemistry of OLIG2 in Grade II Tumors

| Sample no. | Diag | Age | Location of tumor | Positive proportion (%) |
|---|---|---|---|---|
| 11 | AS | 74 | frontal | <1 |
| 12 | AS | 30 | temporal | 7 |
| 13 | AS | 13 | thalamus | 70 |
| 14 | AS | 46 | occipital | <1 |
| 15 | AS | 39 | frontal | 35 |
| 16 | OA | 45 | frontal | 50 |
| 17 | OL | 27 | frontal | 55 |
| 18 | OA | 26 | frontal | 50 |
| 19 | OA | 49 | frontal | 50 |
| 20 | OA | 42 | frontal | 50 |
| 21 | OA | 34 | insular cortex | 50 |
| 22 | OA | 34 | temporal | 75 |

Diag: pathological diagnosis, AS: astrocytoma, OA: oligoastrocytoma, OL: oligodendroglioma.

because their histology was manifold and they were deficient in specific genetic and histologic markers (22). The lineage markers of oligodendrocytes, including NG2, platelet-derived growth factor receptor alpha, and proteolipid protein, have failed to specially distinguish neoplastic oligodendrocytes (23, 24). OLIG2 immunostaining could be of help in the differential diagnosis of AO versus small cell GBM and auxiliary diagnosis of oligodendroglial tumors.

Although the origin of oligodendrogliomas is still controversial, we speculate that a high expression level of OLIG2 in oligodendrogliomas implies that OLIG2 might play an important role in the maintenance of neoplastic oligodendrocytes, which arise from either putative progenitor cells in the adult central nervous system or mature oligodendrocytes. We have shown that OLIG2 was intensely recognized in AO compared with other glial tumors, suggesting that AO might have a certain mechanism to promote OLIG2 expression

TABLE 3
Immunohistochemistry of OLIG2 in Grade III and IV Tumors

| Sample no. | Diag | Age | Location of tumor | Positive proportion (%) |
|---|---|---|---|---|
| 23 | AA | 26 | temporal | 9 |
| 24 | AA | 22 | multiple | 66 |
| 25 | AA | 49 | occipital | 4 |
| 26 | AA | 12 | thalamus | 55 |
| 27 | AA | 73 | front-parietal | 55 |
| 28 | AA | 37 | temporal | 30 |
| 29 | AA | 61 | cerebellum | 30 |
| 30 | AA | 51 | bifrontal | 12 |
| 31 | AA | 56 | frontal | 42 |
| 32 | AO | 63 | frontal | 75 |
| 33 | AO | 73 | parietal | 95 |
| 34 | AO | 26 | frontal | 98 |
| 35 | AO | 29 | temporal | 30 |
| 36 | AO | 75 | insular cortex | 98 |
| 37 | AO | 46 | frontal | 98 |
| 38 | GBM | 51 | front-temporal | 2 |
| 39 | GBM | 22 | frontal | 20 |
| 40 | GBM | 63 | parietal | <1 |
| 41 | GBM | 58 | frontal | 5 |
| 42 | GBM | 72 | parietal | <1 |
| 43 | GBM | 37 | parietal | <1 |
| 44 | GBM | 63 | frontal | <1 |
| 45 | GBM | 43 | temporal | 5 |
| 46 | GBM | 63 | frontal | 3 |

Diag: pathological diagnosis, AA: anaplastic astrocytoma, AO: anaplastic oligoastrocytoma, GBM: glioblastoma.

Interestingly, E proteins, one of the members of the bHLH protein family, regulate cell proliferation and differentiation by forming homodimers or heterodimers with other bHLH proteins. Ectopic expression of the bHLH family of epicardin/capsulin/Pod-1 inhibited E2A-dependent activation of the exogenous and endogenous expression of the cyclin-dependent kinase inhibitor, the p21 (WAF1/Cip1) gene (25). It has been shown that the OLIG protein and E2A protein colocalized in the nuclei of developing oligodendrocytes (26). Thus, OLIG1 and OLIG2, possibly in combination with E2A, may enhance the cell cycle and inhibit differentiation via inhibition of the expression of p21 in neoplastic oligodendrogliomas.

Recently it has been demonstrated that oligodendrogliomas result from the expression of an activated mutant epidermal growth factor receptor in a RAS transgenic astrocytoma model mouse (27). Tumorigenetic progenitor cells of oligodendroglial and astrocytic lineages might express similar biological characters.

In summary, we investigated the expression of *Olig* mRNAs by quantitative RT-PCR on 48 glial tumors. *Olig* transcripts were detected not only in oligodendroglial tumors but also in astrocytic glial tumors. Immunohistochemical analysis revealed that OLIG2 was intensely and ubiquitously expressed in AO, but quite weak in GBM.

This study demonstrated that OLIG2 is not a specific histologic marker for oligodendroglial tumors, however, it might be auxiliary diagnostic tool for the differential diagnosis between AO and GBM. It is necessary to examine more cases for confirmation of the expression of OLIG2 in glial tumors. In addition, the distribution pattern of OLIG2 in a variety of glial tumors may be a clue to the origin of these tumors.

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# Combination therapy of rat brain tumours using localized interstitial hyperthermia and intra-arterial chemotherapy

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Several investigators have reported that a high concentration of drugs in a tumour can be achieved using intra-arterial (IA) chemotherapy. This treatment was highly effective, especially in brain tumours, but the actual therapeutic advantage is still unknown. There are also indications that human malignant gliomas can effectively be treated using interstitial hyperthermia. Therefore, a combined treatment of IA chemotherapy and interstitial hyperthermia should be very promising and this has been studied in a tumour model. Wistar rats with isotransplanted $C_6$ gliomas in the brain were treated with adriamycin (ADR, 1.0 mg/kg body weight) either infused via the carotid artery (i.a.) or via the tail vein (i.v.), with or without interstitial hyperthermia. Hyperthermia of the tumours was applied using a homemade radiofrequency antenna (RF-heating) and a heating device that maintained the tumour temperature above 40°C. Concentration of adriamycin in tumours after treatment was measured using HPLC. The effectiveness of treatment was determined by the survival time of the animals and histopathological examinations. The highest uptake of adriamycin in the rat $C_6$ glioma was obtained when the animals were treated with hyperthermia and i.a. ADR infusion ($p < 0.01$). These animals also showed significantly longer overall survival time (SF50 = 46 days) in comparison to the other treatments ($p < 0.05$). The histological studies demonstrated a necrotic tumour; however, the surrounding normal brain tissue remained intact. Thus, a combination of IA chemotherapy with adriamycin and localized interstitial hyperthermia enhances considerably the efficacy of adriamycin and has a greater antitumour effect for malignant brain tumours. This method is suitable for clinical use, and may be a new strategy for treating gliomas not successfully treated today.

*Key words:* Malignant glioma, hyperthermia, intra-arterial chemotherapy, adriamycin, doxorubicin.

## 1. Introduction

Chemotherapy is one of the adjuvant treatment modalities for human malignant gliomas. Several clinical studies on malignant gliomas with various intra-arterial agents and treatment schedules have demonstrated that intra-arterial (IA) chemo therapy for brain tumours has the advantage of achieving high drug concentrations in the tumour[1], reducing the total dosage of the anti-neoplastic drug and lowering the systemic toxicity[2–9]. However, the therapeutic results are still unsatisfying and the underlying mechanisms are not very well understood.

It is known from clinical and laboratory studies that hyperthermia enhances the cytotoxic effect of several chemotherapeutic drugs[10–22]. Among these is adriamycin

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