

p53 Mutation and Epidermal Growth Factor Receptor Overexpression in Glioblastoma

Recent molecular studies indicate two different genetic pathways leading to the development of glioblastoma; final progression of astrocytoma and de novo formation. To define the mutual relationships of cytogenetic changes in the pathogenesis of glioblastoma, molecular histopathologic alterations of p53 and epidermal growth factor receptor (EGFR) were evaluated by single stranded conformational polymorphism, reverse transcriptase-polymerase chain reaction and immunohistochemical stains in 15 primary and 21 secondary glioblastomas. Mutations in p53 gene and positive immunoreactivity to p53 protein (DO1) were more prevalent in secondary glioblastomas than in primary glioblastomas. A correlation between p53 mutations and p53 immunopositivities in glioblastomas was observed in 83.3% of the cases. All cases with positive p53 immunoreactivities showed p53 mutations; however, 13.9% of glioblastomas with p53 immunopositivities lacked the relevant mutations. EGFR amplifications were detected in 73.3% of primary glioblastomas and 9.5% of secondary glioblastomas ($p < 0.001$). The concurrence of p53 mutation and EGFR amplification was revealed in only 2 out of 15 primary glioblastomas and none among the secondary glioblastomas. Immunoreactivities for EGFR were noted in 66.7% of primary glioblastomas and in 9.5% of secondary glioblastomas ($p < 0.001$). A correlation between EGFR amplification and EGFR immunopositivity in glioblastomas was observed in 91.7% of the cases. These data indicate that EGFR amplification and p53 mutations are two independent genetic events in the development of glioblastomas.

Key Words : Glioblastoma; Protein, p53; Receptor, Epidermal Growth Factor; Immunohistochemistry

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INTRODUCTION

Glioblastoma represents 15-20% of brain tumors and 50% of all gliomas (1). A characteristic histopathologic hallmark of glioblastoma is marked cytologic heterogeneity of astrocytic tumor cells associated with necrosis and/or vascular endothelial proliferation (2, 3). Molecular biologic studies indicate that glioblastoma is the common end point in the progression of grade 2 (low-grade) or grade 3 (anaplastic) astrocytomas (4, 5). In the revised WHO classification of tumors of the central nervous system, glioblastoma is classified under the group of astrocytic tumors, grade 4, as opposed to its prior classification under embryonal tumors (6).

Recent clinical and molecular studies indicate the two different genetic pathways leading to the development of glioblastomas: (a) final progression of lower grade astrocytoma, which arise due to p53 mutation, loss of heterozygosity (LOH) 17p, amplification of MDM2, CDK4, Rb muta-

tion, LOH 10, and epidermal growth factor receptor (EGFR) amplification (7-10); and (b) de novo from rapid malignant transformation of a normal glial cell due to LOH 10 and EGFR amplification (11, 12). Another possible pathway of glioblastoma development is progression from other types of gliomas, such as oligodendrogliomas and oligoastrocytomas by way of genetic lesions of LOH 19q and 1p (13-15), 9p and 10 (16). Common association of EGFR amplification and p53 mutation is very rare in the first pathway (17, 18).

Attempts have been made to link these different molecular genetic pathways in the development of glioblastomas to clinical features (17-20). Secondary glioblastomas derived from lower grade astrocytomas occurs in younger patients (mean age, 40.5 yr), while de novo glioblastomas tend to occur in older patients (mean age, 56.3 yr). However, these studies are primarily based on histopathologic diagnosis of glioblastomas without clinical distinction between primary and secondary etiologies.

The aims of this study are to investigate molecular genetic abnormalities and their correlation to clinical criteria of primary and secondary glioblastomas and to define the mutual relationships between *p53* mutation and EGFR amplification.

MATERIALS AND METHODS

Tumor samples

All tumor specimens were obtained from patients undergoing therapeutic operation for brain tumors at Chonnam National University Hospital from 1993 to 1998. Samples consisted of fresh-frozen tissues and paraffin-embedded tissue blocks, which were selected based on the adequacy for the molecular biologic and immunohistochemical studies.

Of the 36 cases, 15 patients had a clinical history of less than 3 months and were designated as primary glioblastomas. Twenty-one patients had secondary glioblastomas. Previous operations with initial histopathologic diagnosis of low grade astrocytomas and anaplastic astrocytomas were performed in 8 and 13 patients, respectively. The details of the clinical histories are shown in Table 1 and 2.

Immunohistochemistry for *p53* and EGFR proteins

Paraffin-embedded tissue slices, 6 μ m in thickness, were immunostained by the avidin-biotin peroxidase (ABC) method (21). Monoclonal antibodies for DO1 (Santa Cruz, U.S.A.) and EGFR-NCL (Novocastra, UK) were used at a titer of 1:100. DO1 binds to both mutant and wild type *p53* proteins, and EGFR-NCL attaches to the extracellular

ligand-binding domain of EGFR (22). The tissue slices were deparaffinated with xylene. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide in 10% methanol. The tissue slices were hydrated with graded alcohol, treated with 10% normal goat serum for 30 min, and then incubated with primary antibodies overnight at a temperature of 4°C. They were incubated with biotinylated anti-mouse IgG for 30 min at room temperature. The tissue slices for *p53* protein immunostaining were incubated with streptavidin-alkaline phosphatase for 1 hr and developed with fast red TR salt (FRT). EGFR-labelled tissue slices were incubated with 1% avidin-biotinylated horseradish peroxidase in 10% normal goat serum for 1 hr at room temperature and developed in a mixture of 0.4 mg/mL diaminobenzidine (DAB) in 0.1% hydrogen peroxide solution for 40 sec. For negative controls, the primary antibodies were omitted in the process of immunostaining. All immunostained tissues were counterstained with hematoxylin solution for 5 min. After dehydration, the tissue was sealed with a universal mount (Research Genetics, U.S.A.) and examined under a light microscope. Nuclear staining of *p53* was scored semi-quantitatively by counting 1,000 cells in the most prominently stained area of the tissue slides (23). Staining of cell membrane for EGFR was marked as + or - in both low grade and high grade areas of the tissue slides (24).

SSCP analysis for *p53* mutations

DNA was extracted from paraffin sections. For samples with positive immunostaining with DO1 antibody, the same areas were chosen for DNA extraction. For samples with negative immunostaining, DNA was extracted from

Table 1. Clinical data and molecular genetic alterations of cases with primary glioblastomas

Case number	Age (yr)/ Sex	Site	<i>p53</i>		EGFR		
			Mutation*	IHC-LI (%)	Gene amplification	IHCL	IHCH
1	54/M	P	-	0	+	+	+
2	47/M	F	-	0	+	+	+
3	47/M	FP	-	0	-	-	-
4	53/M	F	5/6	3.5	+	+	+
5	46/M	F	-	0	+	+	+
6	39/F	TP	-	1.8	\pm^{\dagger}	-	-
7	35/M	Th	7	2.7	+	+	+
8	48/M	F	-	0	\pm^{\dagger}	-	+
9	68/M	O	-	0	+	+	+
10	53/F	FP	-	0	+	-	+
11	61/F	TP	7	12.3	-	-	-
12	42/F	F	-	8.1	-	-	-
13	56/M	O	-	0	+	+	+
14	55/M	TP	-	0	+	+	+
15	49/F	F	-	0	+	-	-

Sex: M (male), F (female); Site: F (frontal), P (parietal), T (temporal), O (occipital), Th (thalamic)

*: exons, †: equivocal amplification interpreted as-(negative) in case 6, + (positive) in case 8

IHC-LI, labeling index by immunohistochemistry; IHCL, immunohistochemistry in low-grade area; IHCH, immunohistochemistry in high-grade area

Table 2. Clinical data and molecular genetic alterations of cases with secondary glioblastomas

Case number	Age (yr) [†] / Sex	Site	p53		EGFR		
			Mutation*	IHC-LI (%)	Gene amplification	IHCL	IHCH
Secondary glioblastomas developed from low grade astrocytomas							
16	27/36/M	F	8/9	28.6	-	-	-
17	19/38/M	F	-	1.5	-	-	-
18	37/40/F	FP	7	14.5	-	-	-
19	14/33/F	TP	-	0	+	+	+
20	25/34/M	F	7	13.6	-	-	-
21	39/45/F	O	5/6	7.9	-	-	-
22	21/34/M	P	8/9	12.4	-	-	-
23	35/45/M	FP	5/6	3.4	-	-	-
Secondary glioblastomas developed from anaplastic astrocytomas							
24	53/57/M	T	6	30.3	-	-	-
25	39/42/M	F	-	0	-	-	-
26	41/46/M	FP	-	6.5	-	-	-
27	27/34/F	F	7	7.3	-	-	-
28	68/72/M	P	-	8.7	-	-	-
29	41/43/M	PO	8/9	10.5	-	-	-
30	44/47/F	T	-	0	-	-	-
31	50/55/F	F	-	0	+	-	+
32	36/40/F	F	7	1.7	-	-	-
33	23/28/M	FP	8/9	13.9	-	-	-
34	19/27/F	O	7	2.6	-	-	-
35	33/38/M	F	-	5.7	-	-	-
36	33/37/F	TO	6	14.9	-	-	-

*, exons; †, age at the first biopsy of astrocytomas

Sex: M (male), F (female); Site: F (frontal), P (parietal), T (temporal), O (occipital)

IHC-LI, labeling index by immunohistochemistry; IHCL, immunohistochemistry in low-grade area; IHCH, immunohistochemistry in high-grade area

Table 3. The oligonucleotide primers used to amplify exons 4-9 of p53 gene in polymerase chain reaction (5'-3')²⁵

Exon (s)	Upstream	Downstream
4	ATC-TAC-AGT-CCC-CCT-TGC-CG	GCA-ACT-GAC-CGT-GCA-AGT-CA
5/6	TC-CTC-TTC-CTG-CAG-TAC-TC	AGT-TGC-AAA-CCA-GAC-CTC-AG
7	GTG-TTG-CCT-CCT-AGG-TTG-GC	CAA-GTG-GCT-CCT-GAC-CTG-GA
8/9	CCT-ATC-CTG-AGT-AGT-GGT-AT	CCA-AGA-CTT-AGT-ACC-TGA-AG

the lesion which was histologically typical of a glioblastomas, avoiding the peripheral infiltration zone. Primers for exons 4-9 of p53 were synthesized (Life Tec, Korea) according to published sequences (25). Details of primers are shown in Table 3. Polymerase chain reaction (PCR)-single stranded conformational polymorphism (SSCP) analysis was performed on 50 µL of PCR mixture containing PCR reaction buffer (10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100), 10 pmol of each primer, 50 µM dNTPs, 3 µL template DNA, 1 U DNA polymerase, and 0.1 µCi α-³²P dCTP (NEN, U.S.A.).

After adding 20 µL of mineral oil (Sigma, U.S.A.), 35 cycles were executed. Each cycle consisted of denaturation (94°C) for 1 min, annealing for 60 sec (58°C for exons 5/6 and 8/9, and 64°C for exons 4 and 7), and extension for 2 min (72°C), and was carried out in an automated DNA Thermal Cycler (Perkin-Elmer, France). Forty microliter of

stop buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) was added to the PCR product, and 2 µL of the final mixture was sampled.

Samples were heated at 95°C for 5 min and immediately loaded onto a 6% non-denaturing polyacrylamide gel containing 6% glycerol. Gels were run at 30 W for 3 hr, cooled by fan at room temperature, dried at 60°C, and autoradiographed for 12-48 hr.

RT-PCR for EGFR gene amplification

To investigate the expression level of EGFR in human glioblastoma, we employed a reverse transcriptase (RT)-PCR assay using EGFR primers. Total RNA was extracted from the tumor tissues of each glioblastoma patient. After the reverse transcription, EGFR primers were used for cDNA amplification. RNA integrity was confirmed with

Table 4. Summary of clinical data and molecular genetic alterations in 36 patients with glioblastomas

	Primary glioblastoma (n=15)	Secondary glioblastoma (n=21)	<i>p</i> value (Method)
Age at first biopsy (yr)	50.2 ± 8.4*	34.5 ± 12.9*	0.000 (1)
Age at glioblastoma biopsy (yr)	50.2 ± 8.4*	41.5 ± 10.4*	0.009 (1)
Sex ratio (M/F)	10/5(2.0)	12/9 (1.3)	0.411 (2)
<i>p53</i> mutation (SSCP)	20.0%	61.9%	0.014 (2)
<i>P53</i> expression (IHC)	33.3%	81.0%	0.001 (2)
<i>P53</i> labeling index [†]	5.7 ± 4.4%*	10.8 ± 8.3%*	0.002 (1)
<i>EGFR</i> amplification (RT-PCR)	73.3%	9.5%	0.000 (2)
<i>EGFR</i> expression (IHC)	66.7%	9.5%	0.001 (2)
<i>p53</i> mutation and <i>EGFR</i> amplification	13.3%	0.0%	0.000 (2)

n, number of cases; *, Mean ± Standard deviation (S.D.); [†], Mean ± S.D in *P53*-positive cases

SSCP, single stranded conformational polymorphism; RT-PCR, reverse transcriptase polymerase chain reaction; IHC, immunohistochemistry; *EGFR*, epidermal growth factor receptor; (1), Student t-test; (2), chi-square test and Fisher's exact test

parallel RT-PCR amplification using β -actin primers. PCR products were electrophoresed on agarose gels containing ethidium bromide and visualized by UV photography. The detailed procedure was as follows.

Total RNA was extracted from frozen tissues using the LiCl/Urea method (26). For cDNA synthesis, 3 μ g of total RNA was annealed to oligo (dT) 15 in 20 μ L total volume containing 4 μ L of 5 XM-MLV RT buffer (50 mM Tris-HCl, pH 8.3, 7.5 mM KCl, 3 mM MgCl₂, and 10 mM DTT), 0.5 mM dNTP, 20 U RNase and 200 U M-MLV RT (Promega, Germany) and was incubated at 37°C for 1.5 hr. PCR mixture contained 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100), 1 mM MgCl₂, 0.2 μ M of each primer, 0.2 mM dNTP, 2.5 U of Taq DNA polymerase (Promega, Germany) and 1.5 μ g of cDNA in a final volume of 50 μ L. The amplification was performed in the DNA Thermal Cycler. Twenty-five PCR cycles were performed which consisted of 1 min at each temperature (94°C, 53°C, 72°C), except for the initial cycle (5 min at 94°C) and the final extension step (5 min at 72°C). Primer sequences for *EGFR* and β -actin (reference gene) were as follows: 5'-AGCCATGCCCGCATTAGCTC-3' (5' primer) and 5'-AAAGGAATGCAACTTCCCAA-3' (3' primer) for *EGFR* (24) resulting in a 110-bp PCR product, 5'-GACTATGACTTAGTTGCGTTA-3' (5' primer) and 5'-GCCTTCATACATCTCAAGTTG-3' (3' primer) for β -actin resulting in a 501-bp PCR product.

Statistical Analysis

All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS/PC + 7.5, Chicago, IL, U.S.A.). A *p* value of less than 0.05 was accepted as statistically significant. The Student t-test was used to evaluate differences in the age of patients and *p53* labeling index (LI) between primary and secondary glioblastomas. The chi-square and Fisher's exact tests were used to analyze differences in sex ratio, *p53* mutations, *p53* expression, *EGFR* amplification, and *EGFR* expression.

RESULTS

The results of this study are summarized in Table 4. The mean ages of the patients with primary and secondary glioblastomas at the time of surgery were 50.2 ± 8.4 yr and 41.5 ± 10.4 yr respectively. The mean age of the patients with primary glioblastomas was significantly higher than that with secondary glioblastomas. The frequencies of both primary and secondary glioblastoma were higher in males than in females but the difference was not statistically significant.

p53 mutations were detected in 3 out of 15 (20.0%) primary glioblastomas (Fig. 1) and in 13 out of 21 (61.9%) secondary glioblastomas (*p*=0.014). All mutations were located between exons 5 to 9. Immunoreactivity to the *p53* gene product was expressed on nuclei of glioblastoma cells. The distribution of *p53*-positive cells was very heterogeneous in many cases (Fig. 2), and there was no specific rela-

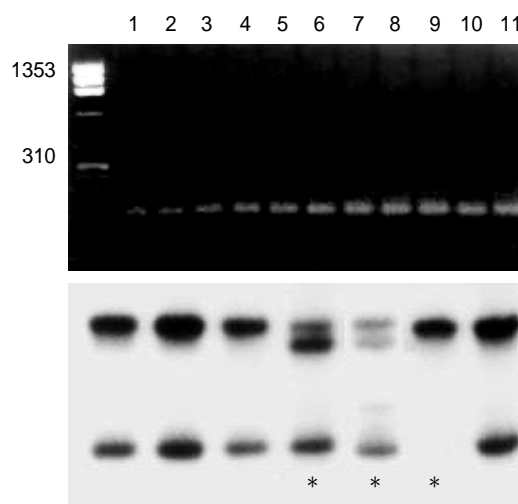


Fig. 1. Presence of exon 7 DNA of the *p53* gene in glioblastomas by PCR (A), and mutations (*) revealed by single stranded conformational polymorphism (Lane 1-11; secondary glioblastoma cases).

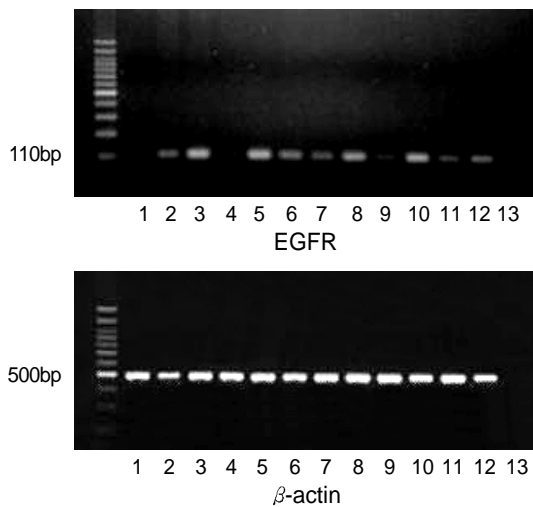


Fig. 2. EGFR amplification detected by reverse transcriptase polymerase chain reaction in a control brain (lane 1), glioblastomas (lanes 2-12), and negative control (lane 13). Note regular 500bp bands of β -actin mRNA as a positive control.

tionship between $p53$ immunoreactivity and nuclear morphology. Immunoreactivity to DO1 was observed in 5 out of 15 (33.3%) primary glioblastomas, and in 17 out of 21 (81.0%) secondary glioblastomas ($p=0.001$). The mean $p53$ labeling index (LI) was $5.7 \pm 4.4\%$ and $10.8 \pm 8.3\%$ in primary and secondary glioblastomas, respectively ($p=0.002$). The correlative results between $p53$ mutations and $p53$ immunoreactivities were observed in 30 out of 36 (83.3%) glioblastomas (both positive, 44.4%; both negative, 38.9%). $p53$ labeling index mutations were observed in all $p53$ -positive glioblastomas; however, 5 out of 36 (13.9%) glioblastomas revealed $p53$ -immunopositivities without $p53$ mutations.

There were two cases with equivocal amplifications of $EGFR$ by RT-PCR. One showed $EGFR$ -positive immunoreactivity and the other was negative. Among the two, the one with positive immunoreactivity to $EGFR$ was considered as positive $EGFR$ amplification. $EGFR$ amplifications were detected in 11 out of 15 (73.3%) primary glioblastomas and 2 out of 21 (9.5%) secondary glioblastomas ($p<0.001$). The concurrence of $p53$ mutation and $EGFR$ amplification was observed only in 2 among 15 primary glioblastomas (Table 1), and even absent in secondary glioblastomas (Table 2). $EGFR$ immunoreactivity was noted along the cell membrane of glioblastoma cells (Fig. 3). Immunoreactivity for $EGFR$ was noted in 10 out of 15 (66.7%) primary glioblastomas, and in 2 out of 21 (9.5%) in secondary glioblastomas ($p<0.001$). The correlative results between $EGFR$ amplification by RT-PCR and $EGFR$ immunoreactivity was observed in 33 out of 36 (91.7%) glioblastomas (both positive, 30.6%; both negative, 61.1%). Only one case revealed $EGFR$ amplification without $EGFR$ -positive

glioblastoma cells. Among the 12 immunopositive cases, nine (75.0%) showed immunoreactivities in both low and high grade areas of glioblastomas while three (25.0%) showed only in high grade areas.

DISCUSSION

The recent molecular biologic studies of astrocytic tumors suggest that both activation of oncogenes and inactivation of tumor suppressor genes are involved in the development of glioblastomas. Von Deimling et al. indicated that there were two types of glioblastomas based on their molecular biologic pathogenesis (27). The type 1 (secondary) glioblastoma occurs with LOH on chromosome 17p closely associated with $p53$ mutations and is more common in younger patients. The type 2 (primary) glioblastoma is characterized by higher frequencies of $EGFR$ amplification and LOH on chromosome 10, and is common in elderly patients. The molecular features of primary and secondary glioblastomas were shown to be strongly correlated with the patients' clinical history in the recent studies (19, 27, 28).

Although some studies support the classification of glioblastomas into two subtypes, only a few studies have analyzed molecular genetic alterations of $EGFR$ and $p53$ in association with immunohistochemical findings on tumor samples. According to our results, the mutations of the $p53$ gene were found in 20.0% of primary glioblastomas and 61.9% of secondary glioblastomas. This observation is consistent with the high rate of $p53$ mutations reported by Watanabe et al. (29). The high rate of $p53$ mutation in secondary glioblastomas might be due to the selection of biopsies according to the definite evidence of clinical progression from low grade or anaplastic astrocytomas. $p53$ protein immunoreactivity was noted in 33.3% and 80.1% of primary and secondary glioblastomas, respectively. In the presence of $p53$ gene mutations, positive staining of tumor cells for $p53$ protein might be expected; however, correlative studies of $p53$ protein immunohistochemistry and $p53$ gene mutation in glioblastomas reported a higher rate of protein expression than the gene mutation (30, 31). The results of this study suggest that an alternative mechanism of $p53$ gene mutation can result in $p53$ protein accumulation. In this study, $p53$ LI was scored $5.7 \pm 4.4\%$ and $10.8 \pm 8.3\%$ in primary and secondary glioblastomas, respectively. There has been reported a wide range of nuclear staining of $p53$ in glioblastomas (23, 29), and the difference might be attributed to the divergent monoclonal antibodies or to the scoring system used in each study.

$EGFR$ is a member of the tyrosine kinase family of cell surface receptors and demonstrates various levels of expression throughout the cellular development and in a variety of different cell types. The receptor can transduce signals into the cells upon: 1) interaction with ligands such as EGF,

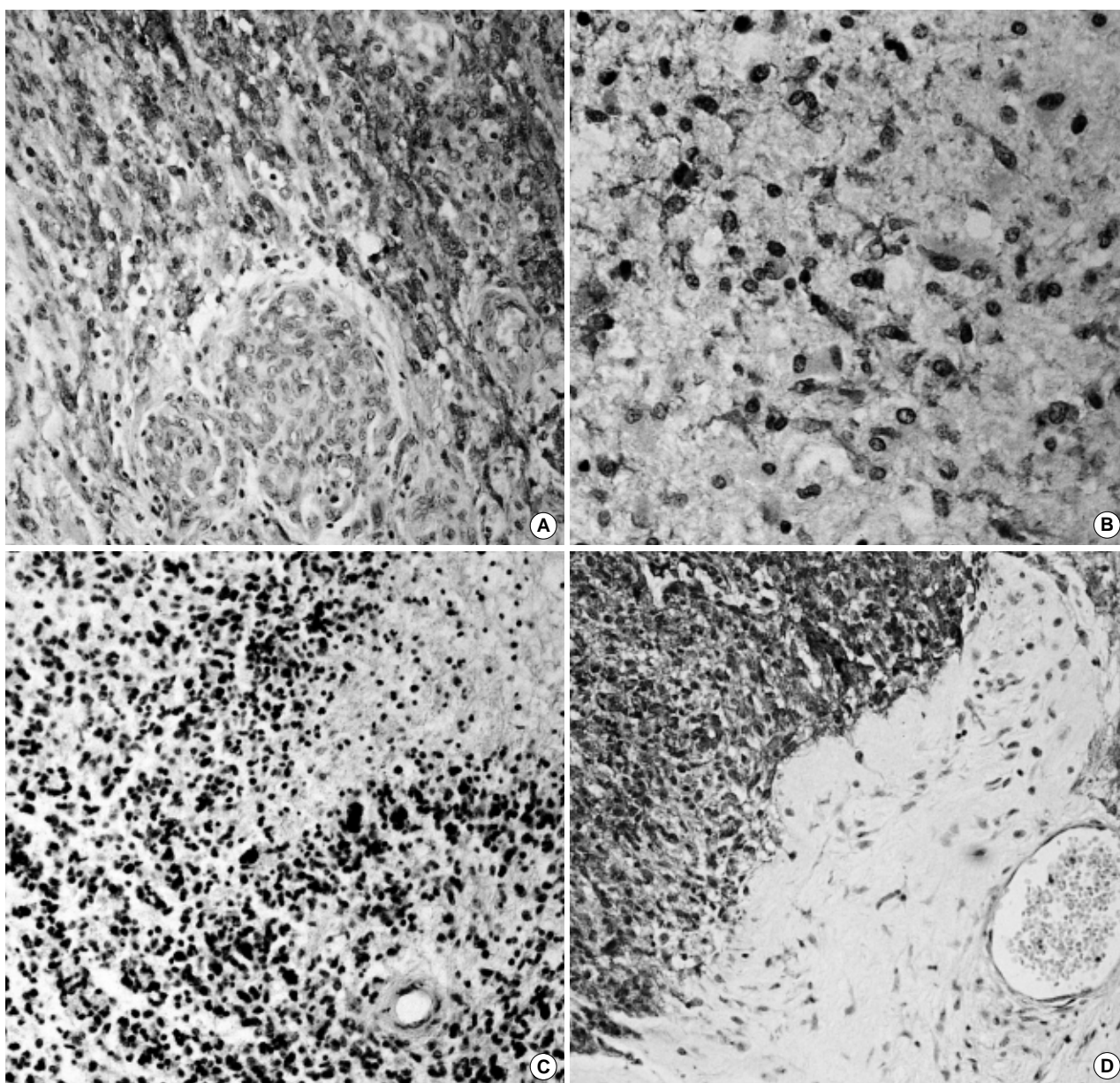


Fig. 3. EGFR immunohistochemistry revealed strong positivity on cell membrane of primary glioblastomas (A: high grade area, B: low grade area) and no immunoreactivity in high grade area (C) in a single case. Similar immunoreactivities are noted in a few cases of secondary glioblastoma (D) (A,B,D \times 100, C \times 90).

transforming growth factor (TGF- α), amphiregulin or heparin-binding EGF; 2) truncation or mutation of extracellular and/or intracellular domains; and 3) amplification of a basal receptor activity in the absence of ligand through cooperation with other cellular signaling pathways or nuclear events, such as an expression of v-erbB (32, 33). EGFR has been implicated in human cancers, where it may contribute both to the initiation (glioblastoma) and progression (epithelial tumors) of the disease. It is frequently present in an amplified or over-expressed form in up to 30-40% of malignant gliomas (34, 35).

According to the present study, the incidence of *EGFR* amplification was 73.3% and 9.5% in primary and secondary glioblastomas, respectively. Recent studies on *EGFR* amplification in glioblastoma were carried out by subgrouping as primary and secondary forms. Lang et al. (36) reported 5 cases out of 34 glioblastomas had *EGFR* amplification and LOH on chromosome 10 without p53 mutations. Watanabe et al. (29) demonstrated a 63% incidence of *EGFR* amplification in primary glioblastomas compared to that of only 10% in secondary glioblastomas. According to the present study, positive immunohistochemical stain-

ings for EGFR were observed in 66.7% and 9.5% of primary and secondary glioblastomas, respectively. Correlative studies between *EGFR* gene amplification and immunohistochemical finding are rare. A comparative study by Rieske et al. (37) reported 13 cases (45%) with immunopositivity for EGFR in a series of 28 glioblastomas, while amplification of *EGFR* gene was observed in 12 cases. In this study, only one case showed no EGFR immunoreactivity in the presence of *EGFR* gene amplification, and two cases demonstrated weak EGFR immunoreactivity without *EGFR* gene amplification. There seems to be no strict correlations between *EGFR* gene amplification and the protein overexpression. EGFR was relatively well expressed in both low grade and high grade areas of glioblastomas, despite a few exceptional cases. Three out of 12 EGFR immunopositive cases were not stained in low grade areas. Concerning with the morphological heterogeneity of glioblastoma, the other study (38) reported that genetic abnormalities seen in the low grade areas were conserved in the high grade areas suggesting that these morphologically different cellular subsets were derived from a common transformed clone. These findings might be helpful to detect the presence or absence of tumor cell infiltrations in the peripheral margin or edematous area of glioblastomas.

In this study, the *p53* mutation with concomitant *EGFR* gene amplification was observed in 13.3% of primary glioblastoma. There were no such cases among secondary glioblastomas. In line with this observation, Watanabe et al. reported only one case with such profile out of 20 secondary glioblastomas and none in 19 primary glioblastomas (29). These data indicate that *EGFR* amplification and *p53* mutation are two different genetic events in the development of glioblastomas.

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