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■ ORIGINAL ARTICLE ■

Immunohistochemical Classification of Primary and Secondary Glioblastomas

Kyu Sang Lee · Gheeyoung Choe[1]
Kyung Han Nam[1] · An Na Seo[1]
Sumi Yun · Kyung Ju Kim
Hwa Jin Cho · Sung Hye Park

Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul; [1]Department of Pathology, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam, Korea

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Corresponding Author
Gheeyoung Choe, M.D.
Department of Pathology, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 82 Gumi-ro 173beon-gil, Bundang-gu, Seongnam 463-707, Korea
Tel: +82-31-787-7711
Fax: +82-31-787-4012
E-mail: gychoe@snu.ac.kr

Background: Glioblastomas may develop *de novo* (primary glioblastomas, P-GBLs) or through progression from lower-grade astrocytomas (secondary glioblastomas, S-GBLs). The aim of this study was to compare the immunohistochemical classification of glioblastomas with clinically determined P-GBLs and S-GBLs to identify the best combination of antibodies for immunohistochemical classification. **Methods:** We evaluated the immunohistochemical expression of epidermal growth factor receptor (EGFR), p53, and isocitrate dehydrogenase 1 (IDH-1) in 150 glioblastoma cases. **Results:** According to clinical history, the glioblastomas analyzed in this study consisted of 146 P-GBLs and 4 S-GBLs. Immunohistochemical expression of EGFR, p53, and IDH-1 was observed in 62.6%, 49.3%, and 11.1%, respectively. Immunohistochemical profiles of EGFR(+)/p53(–), IDH-1(–)/EGFR(+)/p53(–), and EGFR(–)/p53(+) were noted in 41.3%, 40.2%, and 28.7%, respectively. Expression of IDH-1 and EGFR(–)/p53(+) was positively correlated with young age. The typical immunohistochemical features of S-GBLs comprised IDH-1(+)/EGFR(–)/p53(+), and were noted in 3.6% of clinically P-GBLs. The combination of IDH-1(–) or EGFR(+) was the best set of immunohistochemical stains for identifying P-GBLs, whereas the combination of IDH-1(+) and EGFR(–) was best for identifying S-GBLs. **Conclusions:** We recommend a combination of IDH-1 and EGFR for immunohistochemical classification of glioblastomas. We expect our results to be useful for determining treatment strategies for glioblastoma patients.

Key Words: Glioblastoma; Immunohistochemistry; IDH1 protein, human; Genes, erbB-1; Genes, p53

Glioblastomas (World Health Organization [WHO] grade IV) are the most common type of brain tumor and also the most malignant. The prognosis of glioblastomas is very poor, with most patients dying within one year after diagnosis.[1,2] Glioblastomas can be divided into two types, namely, primary and secondary glioblastomas. The German neuropathologist Hans-Joachim Scherer first distinguished primary and secondary glioblastomas in 1940,[3] noting that "from a biological and clinical point of view, the secondary glioblastomas developing in astrocytomas must be distinguished from primary (primary glioblastomas); they are probably responsible for most of the glioblastomas of long clinical duration."[3] In spite of Scherer's remarkable observation, the distinction of primary and secondary glioblastomas has remained conceptual, and has not been used for diagnostic purposes largely because the two types of lesions are histologically indistinguishable. Evidence gained from immunohistochemistry and molecular pathology analysis indicates that primary and secondary glioblastomas constitute distinct disease entities that affect patients at different ages, develop through different molecular pathways, exhibit different genetic expression profiles, and may differ in their response to radiation and chemotherapy.[1]

Primary glioblastomas are also termed *de novo* glioblastomas, and they present as full-blown tumors at diagnosis, and are absent clinical, radiological, or histological evidence of a less malignant astrocytoma. Primary glioblastomas comprise more than 90% of glioblastomas.[1] Secondary glioblastomas develop slowly through progression from a WHO grade II diffuse astrocytoma or WHO grade III anaplastic astrocytoma. On the contrary, the diagnosis of a secondary glioblastoma requires clinical, radiological, or histological evidence of an evolution from a less malignant precursor lesion. Secondary glioblastomas account

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for approximately 5% to 8% of all glioblastomas.[1] The age of onset of secondary glioblastomas is younger than that of primary glioblastomas, and the median survival of secondary glioblastoma patients is 7.8 months, which is significantly longer than that for primary glioblastoma patients (4.7 months, p=0.003). The incidence rate of diffuse and anaplastic astrocytomas is about 2 to 3 times higher than that of secondary glioblastomas, which is reasonable considering the number of patients with diffuse or anaplastic astrocytoma that succumb to the disease before progression to glioblastoma occurs. However, several researchers have suggested that some cases of secondary glioblastomas with very rapid progression from precursor low-grade lesion may be misclassified as primary glioblastomas. By taking into account this possibility, the reported incidence of secondary glioblastomas is likely an underestimate. Nevertheless, secondary glioblastomas constitute a relatively rare disease when compared with primary glioblastomas.[1,4]

In the last two decades, molecular genetic studies have provided considerable insight into the mechanism of tumorigenesis in primary and secondary glioblastomas. Primary glioblastomas typically exhibit epidermal growth factor receptor (EGFR) overexpression, *PTEN* (MMAC1) mutations, *CDKN2A* (p16) deletions, loss of heterozygosity of 10q, and less frequently *MDM2* amplification, whereas *TP53* mutations are early and major genetic alterations leading to secondary glioblastomas.[1,4-7] Watanabe *et al.*[7] emphasized that overexpression of the *EGFR* and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas.

Mutations of isocitrate dehydrogenase (IDH) genes have recently been associated with potential mechanism of glioma pathogenesis.[8-13] IDH-1 and IDH-2 are NADP-dependent enzymes that catalyze the production of α-ketoglutarate from isocitrate during cellular metabolism. Mutations of *IDH-1*, and less frequently *IDH-2*, have recently been identified in glioblastomas, particularly in secondary glioblastomas. *IDH-1* mutations are reported in more than 80% of secondary glioblastomas, whereas they are very rare (1.8%) in primary glioblastomas.[13] The majority of *IDH-1* mutations are observed in combination with either *TP53* mutations or co-deletion of 1p/19q chromosomes, indicating that *IDH-1* mutation are one of the earliest events in the pathogenesis of infiltrating gliomas.[8-13] Moreover, like co-deletion of 1p/19q chromosomes, promoter methylation of methylguanine-DNA methyltransferase (*MGMT*) and EGFR-phosphoinositide 3-kinase pathways as well as *IDH-1* mutations have been demonstrated as important prognostic markers of gliomas. Thus, *IDH-1* mutations in glioblastoma are thought to be closely related to secondary glioblastomas and confer a good prognosis.[8-13]

There is growing interest in the possibility of targeted molecular therapies for malignant tumors.[14,15] In glioblastoma patients, therapeutic response to EGFR tyrosine kinase inhibitors varies significantly according to the expression of EGFR, EGFRvIII, and PTEN.[16-18] Primary and secondary glioblastomas utilize different cell signaling pathways and exhibit distinct patterns of matrix metalloproteinase (MMP) activation.[19,20] In addition, primary and secondary glioblastomas are remarkably different from each other with respect to promoter methylation patterns as well as RNA and protein expression profiles.[1] Therefore, molecular subtyping of glioblastomas are very important for personalized medicine,[21] and distinction of primary and secondary glioblastomas can be the initial step for determining the treatment strategy of glioblastoma patients.

Several laboratory techniques can be employed to distinguish between primary and secondary glioblastomas, with immunohistochemistry, fluorescence *in situ* hybridization (FISH), pyrosequencing, and direct sequencing with polymerase chain reaction comprising the major methods.[14,15] FISH, pyrosequencing, and direct sequencing are more sophisticated techniques; however, these molecular techniques are expensive and time-consuming. In contrast, immunohistochemistry is very fast and economical, and is suitable for screening tests to determine treatment strategies. Indeed, immunohistochemistry is widely utilized in pathology laboratories, and the immunohistochemical evaluation of EGFR and p53 overexpression is routinely available in daily practice for pathologic diagnoses. A recent study reported the usefulness of the immunohistochemical detection of IDH-1 compared to direct DNA sequencing in glioma specimens, demonstrating that there is a statistically significant correlation between the monoclonal antibody immunohistochemistry and the relevant mutation detected by direct DNA sequencing. Thus, IDH-1 immunohistochemistry is useful for evaluating and diagnosing mutation-bearing gliomas as well as for determining treatment strategies.[12,13]

Overexpression of the *EGFR* and p53 mutations are known to be mutually exclusive in the evolution of primary and secondary glioblastomas;[7] however, these two seemingly exclusive pathways do not account for all glioblastomas. In our own practice, we have observed glioblastomas with immunohistochemical co-expression of EGFR and p53, as well as glioblastomas that express neither EGFR nor p53. Thus, the aim of this study was to evaluate the immunohistochemical expression of EGFR, p53, and IDH-1 in a series of 150 glioblastomas in order to

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compare the immunohistochemical classification of glioblastomas with clinically primary and secondary glioblastomas and to determine the best combination of antibodies for their immunohistochemical classification.

MATERIALS AND METHODS

Patients and specimens

We collected formalin-fixed paraffin-embedded tissues from 150 glioblastoma patients. More than one available pathologic examination was available for 10 patients due to tumor recurrence, and for these cases the initial paraffin blocks were used for our study. A total of 118 undergoing craniotomy or stereotactic biopsy in Seoul National University Bundang Hospital from May 2003 to September 2011, and 32 patients received craniotomy or stereotactic biopsy in Seoul National University Hospital from May 2011 to September 2011.

The mean age of 150 glioblastoma patients was 58.8 years with range of 19 to 85 years. The patient group consisted of equal numbers of men and women, 75 patients each. Patients with history of an evolution from diffuse or anaplastic astrocytoma were diagnosed as having clinically secondary glioblastoma, and *de novo* cases were regarded as clinically primary glioblastomas. They consisted of 146 cases of clinically primary glioblastomas and 4 cases of clinically secondary glioblastomas. The mean age of clinically primary and secondary glioblastomas was 59.2 years and 44.0 years, respectively.

Immunohistochemical staining

Immunohistochemical staining for p53 and EGFR was performed in 150 cases using formalin-fixed paraffin-embedded tumor blocks. IDH-1 immunohistochemical stains were performed in 144 cases of glioblastoma for which paraffin blocks were available.

Briefly, 4-μm-thick tissue sections were deparaffinized in xylene and hydrated by immersing in a series of graded ethanol. Antigen retrieval was performed in a microwave by placing the sections in epitope retrieval solution (0.01 M citrate buffer, pH 6.0) for 20 minutes; endogenous peroxidase was inhibited by immersing the sections in 0.3% hydrogen peroxide for 10 minutes.[15] Sections were then incubated with combinations of EGFR (1:150, Dako, Camarillo, CA, USA), p53 (1:1,000, Dako, Glostrup, Denmark), and/or IDH-1 (1:100, DIANOVA, Hamburg, Germany) antibodies.

Immunohistochemical stains for EGFR were graded as follows: 0 (no cell stained), 1+ (<5% tumor cells stained), 2+ (5-50% cells stained), and 3+ (>50% cells stained). For statistical analysis, a score of 0 and 1 was considered negative and a score of 2 or 3 was considered positive. Nuclear staining of p53 was scored semi-quantitatively in the most prominently stained area of the tissue slides. The percentage of positive cells was counted as follows: cases with ≥10% cells were considered positive (overexpression of p53), and <10% cells were considered negative. Cytoplasmic immunoreactivity to the IDH-1 antibody was considered as positive immunostaining.

Histopathological evaluation and radiological review

The hematoxylin and eosin stained slides were reviewed and the diagnosis was confirmed according to the WHO classification of tumors of the nervous system. In cases where the results of immunohistochemical studies were consistent with those of secondary glioblastomas in clinically primary glioblastoma patients with no history of previous diffuse or anaplastic astrocytoma, we searched for histological evidence of an evolution from a diffuse or anaplastic astrocytoma in the background. In addition, we radiologically re-evaluated brain tumor images to detect any lower grade components in the background.

Statistical analysis

All statistical analyses were performed using SPSS ver. 19.0 (SPSS Inc., Chicago, IL, USA). Chi-squared tests were used to analyze correlations between immunohistochemical results. A p-value of less than 0.05 was accepted as statistically significant.

RESULTS

Immunohistochemical expression of EGFR and p53 in glioblastomas

Among 150 cases, immunohistochemical expression of EGFR was noted in 94 cases (62.6%) and p53 was overexpressed in 74 cases (49.3%) (Fig. 1A, B, D, E). The typical immunohistochemical feature of primary glioblastoma, EGFR(+)/p53(−), was noted in 62 cases (41.3%) while the typical immunohistochemical feature of secondary glioblastoma, EGFR(−)/p53(+), was noted in 43 cases (28.7%). Immunohistochemical expression of EGFR(+)/p53(+) was noted in 31 cases (20.7%) and EGFR(−)/p53(−) in 14 cases (9.3%). The inverse correlation between EGFR and p53 was statistically significant (independent samples t-test, $p < .001$).

The mean age of EGFR(+)/p53(−) immunohistochemically primary glioblastoma patients was 65.4 years and the mean age of EGFR(−)/p53(+) immunohistochemically secondary glio-

blastoma patients was 53.8 years. The mean age of EGFR(+)/p53(–) immunohistochemically primary glioblastoma patients was 11.6 years older than that of EGFR(–)/p53(+) immunohistochemically secondary glioblastoma patients, the difference of which was statistically significant (p < .001) (Table 1).

Immunohistochemical expression of IDH-1 in glioblastomas

Immunohistochemical expression of IDH-1 was noted in 16 (11.1%) of 144 total cases (Fig. 1C, F). The mean age of IDH-1(+) glioblastoma patients was 45.2 years, whereas that of IDH-1(–) glioblastoma patients was 60.0 years. Positive IDH-1 status was significantly correlated with young age (p < .001). Among 16 cases of IDH-1(+) glioblastomas, 14 cases showed co-expression of p53 (87.5%) and two cases (12.5%) revealed no overexpression of p53 (p = .01). Among 128 cases of IDH-1(–) glioblastomas, immunohistochemical expression of EGFR was noted in 82 cases (64.1%) (p = .97). We also observed a positive correlation between IDH-1 and p53, but no correlation between IDH-1 and EGFR (Table 2).

Table 1. Immunohistochemical expression of EGFR and p53 in glioblastomas with median age (n = 150)

IHC	No. of cases (%)	Age (yr)	p-value
EGFR(+)	93/150 (62.7)	61.2	.016
p53(+)	74/150 (49.3)	53.5	< .001
EGFR(+)/p53(–)	62/150 (41.3)	65.4	< .001
EGFR(–)/p53(+)	43/150 (28.7)	53.8	.019
EGFR(+)/p53(+)	31/150 (20.7)	53.09	.126
EGFR(–)/p53(–)	14/150 (9.3)	57.9	.075

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry.

Immunohistochemical subtypes in clinically primary and secondary glioblastomas

Clinically secondary glioblastomas were observed in only 4 cases (2.8%) of the 144 cases in our study, which had previously been diagnosed as having diffuse astrocytoma or anaplastic astrocytoma. The proportion of clinically secondary glioblastomas was lower than those of previous studies.[1] The mean age of patients with secondary glioblastomas was 44.0 years. Immunohistochemical profiles of clinically primary and secondary glioblastomas are summarized in Table 3.

All of the clinically secondary glioblastomas expressed IDH-1 according to immunohistochemical staining while none expressed EGFR. The secondary glioblastomas consisted of three

Table 2. Immunohistochemical expression of IDH-1 in glioblastomas (n = 144)

	IDH-1 positive	IDH-1 negative	p-value
No. of cases	16 (11.1)	128 (88.9)	
Age (yr)	45.2	60.0	< .001
Male/Female ratio	1.33	0.56	.136
Clinically secondary GBM	4/4	0/4	.006
EGFR(+)	7/16 (43.8)	82/128 (64.1)	.97
p53(+)	14/16 (87.5)	57/128 (44.5)	.01

Values are presented as number (%).
IDH-1, isocitrate dehydrogenase 1; GBM, glioblastoma; EGFR, epidermal growth factor receptor.

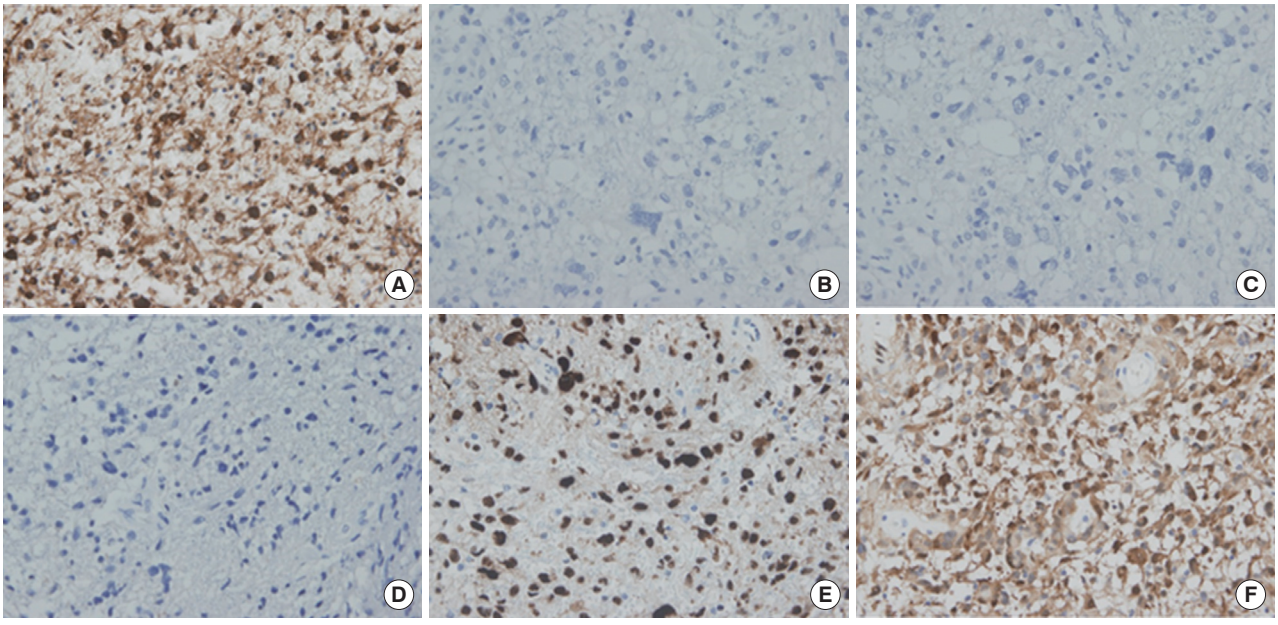

Fig. 1. Characteristic immunohistochemical expression of epidermal growth factor receptor (EGFR), p53, and isocitrate dehydrogenase 1 (IDH-1) in primary glioblastoma (A-C) and secondary glioblastoma (D-F). EGFR (A, D), p53 (B, E), and IDH-1 (C, F).

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cases with an immunohistochemical profile typical of secondary glioblastomas, IDH-1(+)/EGFR(−)/p53(+), and one case of IDH-1(+)/EGFR(−)/p53(−).

Among 140 cases of clinically primary glioblastoma, immunohistochemical profiles typical of primary glioblastomas, IDH-1(−)/EGFR(+)/p53(−), were noted in 58 cases (41.4%). Interestingly, the immunohistochemical profile associated with secondary glioblastomas, IDH-1(+)/EGFR(−)/p53(+), was detected in five cases (3.6%) among the 140 clinically primary glioblastomas. The five patients with IDH-1(+)/EGFR(−)/p53(+) immunohistochemical profiles had no history of lower grade astrocytic tumors; however, they were young with a mean age of 44.6 years. Based on these findings, we requested a neuroradiologist to review the brain magnetic resonance imaging (MRI) images of these five cases. Nonenhancing, extensively infiltrative components without necrosis were detected at least focally in the peripheral areas of the main tumor, which provided radiological evidence of an evolution from a less malignant precursor lesion. In addition, one case presented with multiple lesions as identified by MRI. Based on these findings, the five cases of immunohistochemically secondary glioblastomas were combined with the four cases of clinically secondary glioblastomas. Following this adjustment, the proportion of clinically and immunohistochemically secondary glioblastomas (6.25%) was similar to those of previous studies.[1] Clinicopathologic profiles of clinically and immunohistochemically secondary glioblastoma patients are summarized in Table 4.

Table 3. Immunohistochemical subtypes in clinically primary and secondary glioblastomas (n=144) according to IDH-1, EGFR, and p53 immunohistochemical profiles

IHC	Clinically primary GBM	Clinically secondary GBM
IDH-1(–)/EGFR(+)/p53(–)	58 (40.2)	0 (0)
IDH-1(–)/EGFR(+)/p53(+)	24 (16.6)	0 (0)
IDH-1(–)/EGFR(–)/p53(+)	33 (22.9)	0 (0)
IDH-1(–)/EGFR(–)/p53(–)	13 (9.0)	0 (0)
IDH-1(+)/EGFR(+)/p53(–)	1 (0.6)	0 (0)
IDH-1(+)/EGFR(+)/p53(+)	6 (4.1)	0 (0)
IDH-1(+)/EGFR(–)/p53(–)	0 (0)	1 (0.6)
IDH-1(+)/EGFR(–)/p53(+)	5 (3.4)	3 (2.0)

Values are presented as number (%).
IDH-1, isocitrate dehydrogenase 1; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; GBM, glioblastoma.

Optimal combination of antibodies for immunohistochemical classification of primary and secondary glioblastomas

To determine the best combination of antibodies for immunohistochemical classification of primary and secondary glioblastomas, we analyzed various combinations of antibodies including expression of single individual antibodies, expression profiles of a combination of two antibodies, and expression profiles of all the three antibodies. Specifically, we calculated the statistical measures of the performance of a binary classification tests such as the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value for every antibody combination for identifying immunohistochemically primary glioblastomas (Table 5) and immunohistochemically secondary

Table 4. Clinicopathologic profiles of immunohistochemically secondary glioblastomas

Case No.	Age	Sex	Previous Diagnosis	EGFR	p53	IDH-1
1	36	F	Diffuse astrocytoma	–	–	+
2	39	M	Anaplastic astrocytoma	–	+	+
3	45	M	Diffuse astrocytoma	–	+	+
4	56	F	Anaplastic astrocytoma	–	+	+
5	57	F	None	–	+	+
6	38	M	None	–	+	+
7	69	F	None	–	+	+
8	19	F	None	–	+	+
9	40	M	None	–	+	+

EGFR, epidermal growth factor receptor; IDH-1, isocitrate dehydrogenase 1; F, female; M, male.

Table 5. Statistical measures of the performance of a binary classification test in immunohistochemically primary glioblastomas (n=135) according to different antibody combinations

	Sensitivity	Specificity	Accuracy	PPV	NPV
EGFR(+)	65.93	100.00	68.06	100.00	16.36
p53(–)	53.33	88.89	55.56	98.63	11.27
IDH-1(–)	94.07	100.00	94.44	100.00	52.94
EGFR(+) and p53(–)	43.70	100.00	47.22	100.00	10.59
EGFR(+) or p53(–)	90.37	11.11	85.42	93.85	7.14
IDH-1(–) and EGFR(+)	60.74	100.00	63.19	100.00	14.52
IDH-1(–) or EGFR(+)	100.00	100.00	100.00	100.00	100.00
IDH-1(–) and p53(–)	52.59	100.00	55.56	100.00	12.33
IDH-1(–) or p53(–)	95.56	88.89	95.14	99.23	57.14
EGFR(+) and p53(–) and IDH-1(–)	42.96	100.00	46.53	100.00	10.47

Values are presented as percentage.
PPV, positive predictive value; NPV, negative predictive value; EGFR, epidermal growth factor receptor; IDH-1, isocitrate dehydrogenase 1.

Table 6. Statistical measures of the performance of a binary classification test in immunohistochemically secondary glioblastomas (n = 9) according to different antibody combinations

	Sensitivity	Specificity	Accuracy	PPV	NPV
EGFR(–)	100.00	65.93	68.06	16.36	100.00
p53(+)	88.89	53.33	55.56	11.27	98.63
IDH-1(+)	100.00	94.81	95.14	56.20	100.00
EGFR(–) and p53(+)	88.89	75.56	76.39	19.51	99.03
EGFR(–) or p53(+)	100.00	43.70	47.22	10.59	100.00
IDH-1(+) and EGFR(–)	100.00	100.00	100.00	100.00	100.00
IDH-1(+) or EGFR(–)	100.00	60.74	63.19	14.52	100.00
IDH-1(+) and p53(+)	88.89	95.56	95.14	57.14	99.23
IDH-1(+) or p53(+)	100.00	53.33	56.25	12.50	100.00
EGFR(–) and p53(+) and IDH-1(+)	88.89	100.00	99.31	100.00	99.26

Values are presented as percentage.
PPV, positive predictive value; NPV, negative predictive value; EGFR, epidermal growth factor receptor; IDH-1, isocitrate dehydrogenase 1.

glioblastomas (Table 6).

With respect to immunostaining with a single antibody, expression of p53 exhibited relatively lower sensitivity, specificity, accuracy, positive predictive value, and negative predictive value compared with expression of IDH-1 or EGFR. Contrary to our expectations, the combination of all three antibodies (IDH-1, p53, and EGFR) was not the best way for immunohistochemical classification of primary and secondary glioblastomas. Rather, combination of two antibodies for IDH-1 and EGFR produced the best results in distinguishing primary and secondary glioblastomas. Specifically, combination of IDH-1(–) or EGFR(+) was the best way to identify primary glioblastomas (Table 5), whereas the combination of IDH-1(+) and EGFR(–) was best way to identify secondary glioblastomas (Table 6).

DISCUSSION

The present study consisted of 146 clinically primary and 4 clinically secondary glioblastomas, which had immunohistochemical expression of EGFR, p53, and IDH-1 in 62.6%, 49.3%, and 11.1% of cases, respectively. Immunohistochemical profiles of EGFR(+)/p53(–), IDH-1(–)/EGFR(+)/p53(–), and EGFR(–)/p53(+) were noted in 41.3%, 40.2%, and 28.7% of cases, respectively. In addition, expression of IDH-1 and EGFR(–)/p53(+) was positively correlated with young age. Our study also showed a positive correlation between IDH-1 and p53, but no correlation between IDH-1 and EGFR. Interestingly, 3.6% of clinically primary glioblastomas exhibited the IDH-1(+)/EGFR(–)/p53(+) immunohistochemical profile which is typical of secondary glioblastomas.

Targeted molecular therapies and personalized medicine are becoming increasingly important for the treatment of glioblastoma patients as well as other malignant tumors. Primary glioblastomas are remarkably different from secondary glioblastomas in many aspects, including their therapeutic responses to EGFR tyrosine kinase inhibitors,[16-18] MMP activation,[19] cell signaling pathway,[20] patterns of promoter methylation, and expression profiles at the RNA and protein levels.[1] Because molecular subtyping of glioblastomas is very important for personalized medicine,[21] distinction of primary and secondary glioblastomas can serve as an initial step for determining the treatment strategy of glioblastoma patients.

Amplification/overexpression of *EGFR* is a key pathogenesis in the development of primary glioblastomas. *EGFR* amplification and mRNA overexpression are strongly associated with an increased level of the EGFR protein.[22,23] Increased level of the EGFR protein can be demonstrated as overexpression of EGFR by immunohistochemistry. In previous studies, overexpression of EGFR has been observed in more than 60% of glioblastomas. Likewise in our study, overexpression of EGFR was detected in 62.7% of cases.

The TP53 pathway plays a critical role in the development of low grade gliomas. *TP53* mutations are present in more than 70% of diffuse astrocytomas, anaplastic astrocytomas, and secondary glioblastomas. In addition, *TP53* mutations are noted in lower than 30% of primary glioblastomas.[24] In the presence of *TP53* mutations, positive staining for p53 can be expected; however, a few studies concerning TP53 in glioblastomas have reported a higher rate of immunoreactivity than expected based on the actual gene mutation. In fact, immunohistochemistry does not seem to reflect *TP53* mutations perfectly. Along these lines, several studies have suggested that various mechanisms of TP53 alteration can result in accumulation of p53 protein.[25,26] Similarly, immunohistochemical overexpression of p53 was observed in 49.3% of glioblastomas in our study.

Overexpression of *EGFR* and p53 mutations are known to be

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mutually exclusive in the evolution of primary and secondary glioblastomas.[7] Our study revealed an inverse correlation between EGFR and p53 that was statistically significant ($p < 0.001$); however, 30% of glioblastomas exhibited ambiguous immunoprofiles in our study. The typical immunohistochemical feature of primary glioblastomas, EGFR(+)/p53(–), was noted in 41.3% of cases while the typical immunoprofile of secondary glioblastomas, EGFR(–)/p53(+), was noted in 28.7% of cases. Unexpected immunohistochemical expression of EGFR(+)/p53(+) and EGFR(-)/p53(–) was noted in 20.7% and 9.3% of cases, respectively. Thus, our attempt to classify glioblastomas by the immunohistochemical combination of EGFR and p53 revealed certain limitations.

Because of the inability of EGFR and p53 staining to fully distinguish between clinically primary and secondary glioblastomas, we included immunohistochemical staining for IDH-1, which is highly related to secondary glioblastoma.[8] We then analyzed the expression profiles of triple markers (IDH-1, EGFR, and p53) with respect to classification of glioblastomas (primary vs secondary). Three out of four cases of clinically secondary glioblastomas exhibited the typical immunohistochemical profile consisting of IDH-1(+)/EGFR(–)/p53(+), while the typical immunoprofile of primary glioblastoma, IDH-1(-)/EGFR(+)/p53(–), was noted in 41.4% cases. Interestingly, the typical immunoprofile of secondary glioblastoma, IDH-1(+)/EGFR(–)/p53(+), was detected in five cases (3.6%) among 140 clinically primary glioblastomas. The five patients with IDH-1(+)/EGFR(–)/p53(+) immunoprofile had no history of lower grade astrocytic tumors; however, they were observed in younger patients with a mean age of 44.6 years and presented with diffuse or multiple lesions on MRI images. Therefore, these cases were reclassified as immunohistochemically secondary glioblastomas. We suspect that these cases consisted of secondary glioblastomas with subclinical diffuse or anaplastic astrocytomas and very rapid progression from precursor lower grade lesions.

The proportion of clinically secondary glioblastomas (2.8%) observed in our study was lower previously observed.[1] However, the proportion of clinically and immunohistochemically secondary glioblastomas (6.25%) was similar to those of previous studies. The mean age of secondary glioblastomas was 44.3 years (44.0 years in clinically secondary glioblastomas and 44.6 years in immunohistochemically secondary glioblastomas). Conversely, the mean age of clinically primary and immunohistochemically primary glioblastomas was 59.2 years and 59.3 years, respectively.

To determine the best combination of antibodies for immunohistochemical classification of glioblastomas, we evaluated the statistical performance of a binary classification test using every combination of antibodies for immunohistochemically primary and secondary glioblastomas. Contrary to our expectations, the combination of all three antibodies (IDH-1, p53, and EGFR) was not the best way to distinguish primary and secondary glioblastomas. Rather, combinations of IDH-1 and/or EGFR performed better for distinguishing primary and secondary glioblastomas. Specifically, the combination of IDH-1(+) and EGFR(–) was the best way to identify secondary glioblastomas, which exhibited perfect statistical significance for sensitivity, specificity, accuracy, positive predictive value, and negative predictive value. Likewise, the immunohistochemical combination of IDH-1(–) or EGFR(+) was the best method for identifying primary glioblastomas. In other words, an IDH-1(+)/EGFR(–) immunoprofile accurately identified secondary glioblastomas, while IDH-1(–)/EGFR(+), IDH-1(+)/EGFR(+), and IDH-1(–)/EGFR(–) immunoprofiles identified primary glioblastomas. Based on these results, the combination of IDH-1 and EGFR immunohistochemistry, excluding p53, was determined to be a good method for subtyping glioblastomas. This result was attributed primarily to one case of the four clinically secondary glioblastomas that did not express p53, which affected the statistical significance of the data considerably. Moreover, overexpression of p53 was frequently observed in primary glioblastomas. The sensitivity of p53(–) was too low (53.3%) in primary glioblastomas, and thus the immunohistochemical overexpression of p53 was not statistically useful for distinguishing primary and secondary glioblastomas.

In summary, immunohistochemical expression of EGFR, p53, and IDH-1 in glioblastomas was observed in 62.6%, 49.3%, and 11.1%, respectively. The immunohistochemical profiles of EGFR(+)/p53(–), IDH-1(–)/EGFR(+)/p53(–), and EGFR(–)/p53(+) were noted in 41.3%, 40.2%, and 28.7% of cases, respectively. In addition, expression of IDH-1 and EGFR(–)/p53(+) was positively correlated with young age. Our study demonstrated a positive correlation between IDH-1 and p53, but not between IDH-1 and EGFR. The typical immunoprofile of secondary glioblastomas, IDH-1(+)/EGFR(–)/p53(+), was detected in 3.6% of clinically primary glioblastomas. The combination of IDH-1(–) or EGFR(+) was the best method for identifying primary glioblastomas, whereas the combination of IDH-1(+) and EGFR(–) was the best method for identifying secondary glioblastomas.

In conclusion, we recommend the combination of IDH-1

and EGFR for immunohistochemical classification of glioblastomas. We expect that our results will be useful for determining treatment strategies for patients with glioblastoma.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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