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## BRAF Overexpression Induces Rampant Glioma Proliferation Independent of Phospho-EGFR Expression<sup>\*\*</sup>

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;  
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### Abstract

**Background.** The prognosis for gliomas is still dismal when treated with traditional approaches. Molecular targeted therapy has become a trend in treating tumors, including gliomas. However, molecular characteristics vary among different kinds of tumors and ethnic groups.

**Objectives.** The aim of the research was to study the expression characteristics of key components in the EGFR pathway of gliomas in order to contribute new data for the molecular targeted treatment of gliomas.

**Material and Methods.** EGFR, KRAS, BRAF, PI3K, phospho-EGFR and Ki67 expression were detected with immunohistochemistry in 82 glioma specimens.

**Results.** The expression of EGFR was positively correlated with the patients' age. There were no significant differences in clinicopathological characteristics between gliomas with and without phospho-EGFR expression. EGFR overexpression was significantly correlated with phospho-EGFR expression. In gliomas with EGFR activation, overexpressions of EGFR, BRAF and PI3K were significantly correlated with proliferation. However, in gliomas without phospho-EGFR expression, only BRAF overexpression was significantly related to the proliferation of tumor cells.

**Conclusions.** BRAF overexpression could be an independent factor causing tumorigenesis in gliomas regardless of phospho-EGFR expression. The molecular characteristics can vary with the increasing age of glioma patients (**Adv Clin Exp Med 2014, 23, 6, 893–899**).

**Key words:** glioma, molecular targeted therapy, EGFR protein, BRAF protein, immunohistochemistry.

Gliomas, which arise from glial cells, are the most common tumor of the central nervous system. They are characterized by rampant proliferation and recurrence. Despite the availability of traditional treatment options such as surgical resection, radiotherapy and traditional chemotherapy, the prognosis is often dismal. Recently, molecular targeted drugs have become widely used in treating malignant tumors [1]. It has been proven that some drugs can significantly prolong survival and improve the quality of life by specifically blocking signal transduction in tumor cells to

inhibit their growth or suppress new blood vessel formation [2]. Additionally, unlike traditional chemotherapeutic drugs, molecular targeted drugs are more effective in treatment as they focus on specific molecular changes, unrelated to the tumor's tissue of origin. This makes it possible to treat gliomas effectively by selecting molecular targeted drugs that address some specific molecular changes [3, 4]. Therefore, it has become necessary to better understand the molecular characteristics of glioma, in addition to their histopathological and morphological characteristics.

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Aberrant activation of the EGFR signal transduction pathway plays a pivotal role in the tumorigenesis of many malignant tumors, including gliomas [5]. Activated EGFR can initiate downstream signaling cascades, primarily in the KRAS/BRAF/ERK/MAPK pathway, and also in the PI3K/AKT pathway, thereby pushing cells into the process of proliferation [5, 6]. The EGFR family, which are receptor tyrosine kinases, is the key molecular node of signal transduction, in which spontaneous activation is the most common event in malignant tumors. However, the initial agent of activation of the EGFR pathway varies among different tumors. It has been reported that Her-2 amplification or overexpression is often found in breast cancer [7]. EGFR mutation in the intracellular domain (mainly in exon 19 and 21) is often seen in non-small cell lung cancer (NSCLC), while in gliomas, EGFR mutation is mainly focused in the extracellular domain (EGFRvIII) [8, 9]. Besides EGFR, any other component of its downstream signaling that is activated abnormally can also cause cells to proliferate malignantly. KRAS, BRAF and PI3K mutations can activate the EGFR pathway, as found in colorectal carcinoma, thyroid carcinoma and melanoma, among others [10–12]. In prostate tumors, the PI3K signal pathway can be activated by PI3K overexpression or amplification, but not by its mutation [13].

Interestingly, even in the same kind of tumor, the cause of activation of the EGFR pathway varies among ethnic groups. The frequency of KRAS mutations was found to be about 30% and the frequency of EGFR mutation was less than 10% in NSCLC patients from Western countries [14, 15]; in contrast, the frequency of KRAS mutation was found to be less than 10%, while the frequency of EGFR mutation was 30–50% in NSCLC patients from East Asia [16, 17]. There is still uncertainty about the genetic alterations that occur in the genes of the EGFR signal transduction pathway in gliomas. The authors hope to contribute new data from glioma patients in China.

It has been reported that EGFR overexpression, amplification and/or mutation (exon 2 to 7 deletion) are present in nearly half of all gliomas [18]. Although the abnormal activation of EGFR is an important factor in glioma tumorigenesis, there are other gliomas that proliferate without EGFR activation. Reports on the characteristics of gene alterations in gliomas without EGFR activation are few in number. EGFR activated in any way signals downstream by phosphorylation in the intracellular domain of EGFR. Therefore, phospho-EGFR expression was used in this study as the marker for EGFR activation, and KRAS, BRAF, PI3K, Ki67

expression were detected simultaneously, in order to analyze the molecular characteristics of the EGFR signaling pathway in gliomas and better understand its clinical significance.

## Material and Methods

### The Patients and Tumor Specimens

In this study, 82 patients who were diagnosed with primary gliomas and treated with surgical resection at the Second Hospital of Dalian Medical University between 2001 and 2008 were selected continuously. None had radiotherapy or chemotherapy before surgery. Out of the total, 42 cases were female and 40 male, and they included 5 patients under 18 years old (4 girls and 1 boy). The ages of the patients ranged from 3 to 75 years, with a mean  $\pm$  SD age of  $46.4 \pm 17.5$  years. There were 64 gliomas occurring in the telencephalon (35 in the right cerebral hemisphere and 31 in the left cerebral hemisphere, including 32 in the frontal lobe, 8 in the parietal lobe, 13 in the temporal lobe, 3 in the occipital lobe, 5 in the frontal and temporal lobe, 1 in the frontal and parietal lobe, 2 in the parietal and occipital lobe), 6 gliomas in the cerebellum, 1 glioma in the *corpus callosum*, 1 glioma in the pineal body, 1 glioma in the saddle area, 2 gliomas in the lateral ventricle, 1 glioma in the ventricle, 1 glioma in the brain stem and 5 gliomas in the spinal cord. After surgical removal the tumor specimens were incubated in 10% neutral-buffered formalin and paraffin embedded. All the tumor specimens were diagnosed and classified by 2 licensed pathologists as grades I–IV according to the WHO classification system, including 4 grade I gliomas (all pilocytic astrocytomas), 28 grade II gliomas (consisting of 19 astrocytomas, 1 oligodendroglioma, 4 oligoastrocytomas and 4 ependymomas), 24 grade III gliomas (19 anaplastic astrocytomas, 4 anaplastic oligoastrocytomas and 1 anaplastic ependymoma), and 26 grade IV gliomas (24 glioblastomas and 2 medulloblastomas). All the specimens collected with the approval of the Ethics Committee of the Second Hospital of Dalian Medical University and with the informed consent of the patients before surgery.

### Immunohistochemistry

Monoclonal antibodies against EGFR (1 : 100 dilution; clone 111.6; Thermo Fisher Scientific, USA), KRAS (1 : 20 dilution; clone F 132-62; Abcam), BRAF (1 : 500 dilution; clone F-7; Santa Cruz Biotech), PI3K (1 : 200 dilution; clone SP62; Spring

Bioscience), phospho-EGFR (1 : 1600 dilution; clone 1H12; Cell Signaling Technology) and Ki67 (1 : 100 dilution; clone K-2; Life Technologies) were used as primary antibodies. Immunohistochemistry was performed according to a previously described protocol [19]. Two cases failed to detect KRAS, and 10 cases failed to detect PI3K. As the negative control, phosphate buffered saline (PBS) was used to replace the primary antibody. The tumor cell was considered positive when there were brown granules in the membrane and cytoplasm, detecting EGFR and phospho-EGFR; when brown granules were only in cytoplasm, detecting KRAS, BRAF and PI3K; and when they were in the nucleus, detecting Ki67. The immunoreactivity of EGFR, KRAS, BRAF and PI3K was evaluated as negative when < 25% of the tumor cells showed positive; as weakly positive when 25–75% of the tumor cells showed positive; and as strongly positive when  $\geq 75\%$  of the tumor cells showed positive. For phospho-EGFR, the immunoreactivity was evaluated as negative when < 25% of the tumor cells showed positive and positive when  $\geq 25\%$  of the tumor cells showed positive. For Ki67, the immunoreactivity was evaluated as negative when < 1% of the tumor cells showed positive; weakly positive when 1–10% of the tumor cells showed positive; and strongly positive when  $\geq 10\%$  of the tumor cells showed positive. All the staining results were evaluated by two independent pathologists.

## Statistical Analysis

The Pearson chi-square test and Fisher's exact test were used to compare the differences in positive expression frequencies between groups classified according to their clinicopathological characteristics. Spearman's rank-order correlation test was used to assess the relationships among different protein expression levels and the relationships of these proteins' expression levels to the patients' age and WHO classifications. All statistical analyses were performed with SPSS 15.0 software. Significance was set at the 0.05 level (2-tailed).

## Results

### EGFR, KRAS, BRAF, PI3K, Ki67 and Phospho-EGFR Expression in Gliomas

Out of the total of 82 gliomas, positive expression frequencies were 59.8% (49/82) for phospho-EGFR; 75.6% (62/82, including 38 weak positives and 24 strong positives) for EGFR; 63.8% (51/80, including 27 weak positives and 24 strong

positives) for KRAS; 72.0% (59/82, including 18 weak positives and 41 strong positives) for BRAF; 68.1% (49/72, including 16 weak positives and 33 strong positives) for PI3K; 69.5% (57/82, including 41 weak positives and 16 strong positives) for Ki67 (Fig. 1).

The patients were divided into 3 groups according to their ages: children under 18 years, adults 18–50 years, and adults over 50 years. The ages of the glioma patients were positive correlated with the grades of the tumors ( $p = 0.003$ ). The gliomas in children were mainly Grade II (4/5); those in the young adult patients (18–50 years) were mainly Grades II (17/42) and III (16/42); while those in the older adult patients (over 50 years) were mainly Grade IV (18/35).

The relationship of all the proteins' expression to the histopathological characteristics is presented in Table 1. The frequencies of EGFR, BRAF and Ki67 expression were all significantly higher in adult glioma patients than in child patients ( $p < 0.05$ ). The expression of EGFR was positively correlated with age levels ( $p = 0.014$ ,  $r = 0.270$ ), while those of other proteins were not. The expression of Ki67 was positively correlated with WHO classifications ( $p < 0.0005$ ,  $r = 0.458$ ), while those of other proteins were not. None of frequencies of the proteins' expression differed significantly between genders or between tumor sites (left and right cerebral hemisphere).

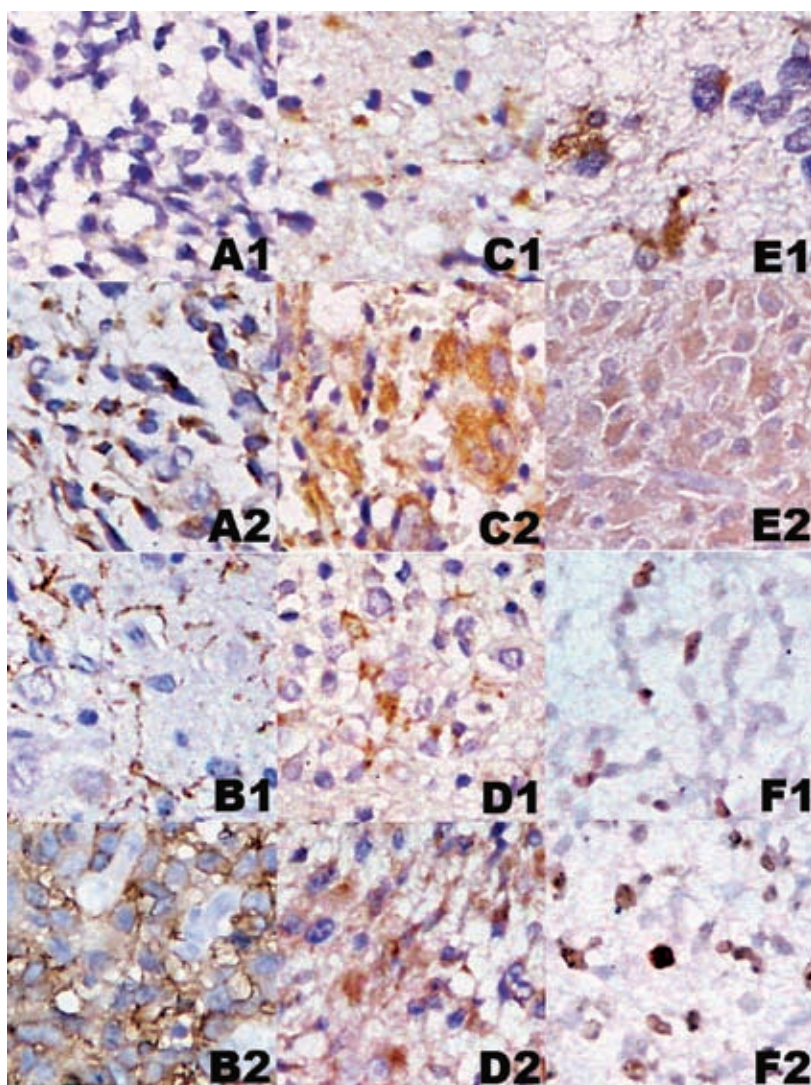
### Clinicopathological Characteristics of Gliomas with and Without Phospho-EGFR Expression

Phospho-EGFR expression was detected as the marker of EGFR activation. All the gliomas were divided into 2 groups: those with phospho-EGFR expression (49 cases) and those without phospho-EGFR expression (33 cases). There were no significant differences in the ages and genders of patients or in tumor sites between the two groups ( $p > 0.05$ ).

### Molecular Characteristics of Gliomas with and Without Phospho-EGFR Expression

The frequency of EGFR expression was significantly higher in gliomas with phospho-EGFR expression than in those without it ( $p = 0.029$ ). There were no significant differences in the frequencies of KRAS, BRAF, PI3K or Ki67 between the 2 groups ( $p > 0.05$ ). In gliomas with phospho-EGFR





**Fig. 1.** Protein expression detected by immunohistochemistry, A1 – phospho-EGFR expression was negative; A2 – phospho-EGFR expression was positive. B, C D, E and F were EGFR, KRAS, BRAF, PI3K and Ki67 expression, respectively. 1 was weakly positive and 2 was strongly positive ( $\times 200$ )

**Table 1.** The relationships between clinicopathological characteristics and the expression of phospho-EGFR, EGFR, KRAS, BRAF, PI3K and Ki67

	Cases	Phospho-EGFR	EGFR	KRAS*	BRAF	PI3K**	Ki67
Gender							
Male	40	24 (60.0%)	30 (75.0%)	28 (71.8%)	30 (75.0%)	26 (76.5%)	31 (77.5%)
Female	42	25 (59.5%)	32 (76.2%)	23 (56.1%)	29 (69.0%)	23 (60.5%)	26 (61.9%)
Age							
Children ( $\leq 18$ )	5	3 (60.0%)	1 (20.0%)#	2 (40.0%)	1 (20.0%)#	2 (50.0%)	1 (20.0%)#
Adults ( $>18$ )	77	46 (59.7%)	61 (79.2%)	49 (65.3%)	58 (75.3%)	47 (69.1%)	56 (72.7%)
WHO classification							
Grade I–II	32	19 (59.4%)	22 (68.8%)	21 (67.7%)	20 (62.5%)	17 (60.7%)	13 (40.6%)
Grade III–IV	50	30 (60.0%)	40 (80.0%)	30 (61.2%)	39 (78.0%)	32 (72.7%)	44 (88.0%)
Site							
Left cerebral hemisphere	35	21 (60.0%)	29 (82.9%)	22 (64.7%)	27 (77.1%)	25 (78.1%)	28 (80.0%)
Right cerebral hemisphere	29	16 (55.2%)	22 (75.9%)	16 (57.1%)	21 (72.4%)	16 (66.7%)	20 (69.0%)

\* – two cases failed to detect KRAS; \*\* – ten cases failed to detect PI3K; # – there were significant differences between child and adult patients ( $p < 0.05$ ).

**Table 2.** Relationships among EGFR, KRAS, BRAF, PI3K and Ki67 expressions and WHO classifications in gliomas with and without phospho-EGFR expression

	KRAS	BRAF	PI3K	Ki67	WHO classifications
EGFR		0.437*		0.321*	
KRAS			0.509#		
BRAF				0.371*	0.392#
PI3K					0.462*
Ki67					0.533*, 0.369#

Spearman's rank-order correlation was used to separately analyze the relationship between protein expression levels and WHO classifications in gliomas with and without phospho-EGFR expression.

\* – was used to mark the correlation coefficients in gliomas with phospho-EGFR expression ( $p < 0.05$ ).

# – was used to mark the correlation coefficients in gliomas without phospho-EGFR expression ( $p < 0.05$ ).

expression, EGFR expression was found to be positively correlated with BRAF expression ( $p = 0.002$ ). Expression of EGFR and BRAF were both positively correlated with Ki67 expression ( $p = 0.024$  and  $p = 0.009$ , respectively); and PI3K expression was positively correlated with tumor grade ( $p = 0.001$ ) (Table 2). In gliomas without phospho-EGFR expression, KRAS expression was positively correlated with PI3K expression ( $p = 0.007$ ); and BRAF expression was positively correlated with tumor grade ( $p = 0.024$ ). However, expression of EGFR, KRAS, BRAF and PI3K were not significantly correlated with either Ki67 expression or with tumor grade (Table 2).

## Discussion

EGFR is a transmembrane receptor tyrosine kinase, and phosphorylation of EGFR will cause EGFR pathway activation. Aberrant EGFR is the key component activating the EGFR signal transduction pathway in many malignant tumors [5, 6, 20]. Gene mutation (including mutation in the extracellular and intracellular domain), amplification and overexpression are common ways EGFR is activated abnormally in gliomas [18, 21]. Therefore, the authors of this study selected monoclonal anti-EGFR, which can bind the extracellular domain of EGFR whether it is activated (in any way) or inactivated, to detect EGFR expression, and the anti-phospho-EGFR to indicate the state of EGFR activation. No differences were found between gliomas with and without phospho-EGFR expression in relation to clinicopathological characteristics such as the patients' gender and age or the tumor grade. KRAS, BRAF, PI3K and Ki67 expression did not vary with these characteristics. But it was found that the expression frequency of EGFR was significantly higher in gliomas with phospho-EGFR expression than in those without its expression.

Moreover, frequency was higher as the patients' age increased, and it was significantly higher in adult patients as compared to children. It is thought that the distribution of the 4 grades of glioma varies with the patient's age. Pilocytic astrocytoma (Grade I) and pleomorphic xanthoastrocytoma (Grade II) are commonly found in children, while glioblastoma multiforme (Grade IV) is most often found in patients between 45 to 70 years [22]. In the present study it was also found that gliomas often had higher grades in older patients. This suggests that aging could be related to a more malignant histological type of glioma, which means the tumor cells have a stronger proliferation ability. It is not strange that aging is also related to EGFR overexpression in gliomas ( $p = 0.014$ ,  $r = 0.270$ ). However, phospho-EGFR expression didn't increase with age. This indicates that in gliomas from adult patients, EGFR overexpression could not be the reason for abnormal EGFR activation.

Gliomas are different from most other malignant tumors. Metastasis is not common, but rampant proliferation is a main feature of gliomas and is closely related to the prognosis. The higher the grade of the glioma, the stronger its ability to proliferate, and the poorer the prognosis of the patient [23, 24]. Ki67 is the most common molecular marker indicating the ability to proliferate in gliomas [25]. The percentage of Ki67 expression in glioma cells reflects the potential ability to proliferate and is related to prognosis. Although the frequency of Ki67 expression overlaps in different histological types of gliomas, it is mainly 5–10% in Grade III gliomas and  $< 5\%$  in Grade II gliomas [30, 31]. Therefore, in the present study 1% and 10% were selected as the cut-off values, in order to clearly indicate the proliferation ability of gliomas. Ki67 expression has usually been shown to be significantly related to the grade of the glioma, and this was supported by the results of the present study. In gliomas with phospho-EGFR

expression, EGFR expression was found to be significantly related to BRAF expression. At the same time, EGFR and BRAF expression were significantly correlated with separate Ki67 expression in gliomas, and PI3K expression was significantly correlated with tumor grade. This suggests that either EGFR, BRAF or PI3K overexpression could activate the EGFR signal pathway independently. There could be multi-activating components participating in the rampant proliferation in gliomas with EGFR activation. In this situation, the use of combined modality therapy with EGFR, BRAF and PI3K inhibitors should be considered.

The PI3K/AKT signal pathway is often thought to be peripherally downstream of EGFR/KRAS. This pathway also regulates cell proliferation, differentiation and anti-apoptosis [6, 26]. When the EGFR/KRAS/BRAF/ERK pathway is blocked, the PI3K/AKT pathway would be activated, and this was often thought to be a mechanism of resistance to EGFR tyrosine kinase inhibitors in NSCLC [27]. Aberrant PI3K activation would also activate the PI3K/AKT pathway [28]. It has been reported that PI3K mutation and amplification have been found in some gliomas [29]. In this

study, KRAS expression was found to be significantly correlated with PI3K expression in gliomas without phospho-EGFR expression. This suggests that the PI3K/AKT pathway participates in the tumorigenesis of gliomas without EGFR activation. Interestingly, it was BRAF expression, not that of PI3K expression, that was related to the grades of gliomas without phospho-EGFR expression. This suggested that BRAF overexpression could activate the BRAF signal pathway independently and then cause rampant proliferation in gliomas even without phospho-EGFR expression.

In conclusion, BRAF overexpression could be an independent factor that causes tumorigenesis in gliomas either with or without phospho-EGFR expression. EGFR overexpression could be a result of increasing age. This should be considered carefully with regard to glioma patients treated only with EGFR inhibitors.

A limitation of the current study was that just one method of immunohistochemistry and one monoclonal antibody against phospho-EGFR were used. Further study is required to provide further proof of the possibility of BRAF as a molecular target in treating gliomas.

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