

RESEARCH ARTICLE

Expression of EGFR in Paired New and Recurrent Glioblastomas

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Abstract

Background: The aim of this study was to analyse the expression of EGFR in newly diagnosed and recurrent glioblastoma multiforme (GBM). **Materials and Methods:** Our study included a total of 48 paired samples collected from 24 patients diagnosed with GBM. The intensity of EGFR cytoplasmatic staining was scored on a scale of 1–3+ (weak, intermediate or strong). **Results:** We found EGFR overexpression in 23 patients (96%) with newly diagnosed GBM, while all recurrent tumours overexpressed EGFR. Ten recurrent tumours (42%) had a lower expression than their new counterpart 13 tumours (54%) had a similar expression, and only one case (2%) had increased expression on recurrence. The expression of EGFR in newly diagnosed GBM was significantly correlated with EGFR expression in recurrent tumour ($p = 0.036$). In addition, new GBMs with strong EGFR expression had a mean relapse-free interval of 11.5 months ($p=0.017$). A benefit of combined therapy was observed in the radiotherapy-plus-chemotherapy group where the average time was 11 months ($p=0.011$), as compared with surgery/radiotherapy alone (average time 6.8 months). **Conclusions:** The present data show that EGFR is overexpressed in paired GBMs. The discrepancies of EGFR expression between the primary tumour and the recurrence suggest heterogeneity of GBMs but also unity at relapse.

Keywords: Epidermal growth factor receptor - glioblastoma - immunohistochemistry - paired tumours

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Introduction

Cerebral tumours are characterized by one of the most difficult oncological management strategies with variability depending on the histopathological identity of each neoplastic entity. Tumours such as grade IV astrocytoma, nominated as glioblastoma multiforme (GBM), are considerable therapeutically challenge with no definite treatment nowadays and with a mean survival of approximately 15 months (Ohgaki and Kleihues, 2005; Nana et al., 2015). Despite the standard treatment (surgical treatment, chemotherapy and radiotherapy), GBM usually recurs decreasing the median survival to only five to seven months (Henriksson et al., 2011). Surgical resection is rarely performed at recurrence, and there is a lack of data regarding pathological and molecular features of relapsed GBMs.

Overexpression and amplification of the epidermal growth factor receptor (EGFR) were found to play a significant role in the development and progression of GBM, (Vivanco et al., 2012; Nana et al., 2015). Given the fact that more than 50% of GBMs involve alterations of EGFR, new therapeutic avenues are being investigated

in the light of molecular and cellular advancements that are targeting EGFR (Heimberger et al., 2005; Gallego et al., 2014; Azuaje et al., 2015). Studies elaborate different variations of EGFR expression in GBM, but it is unclear the situation of these alternations in the biology of the relapsed tumours. The purpose of our study was to evaluate the differences between the newly GBM and its recurrence through the spectrum of EGFR expression.

Materials and Methods

Patients

Our study included a total of 48 paired samples collected from 24 patients diagnosed with GBM, between 2010-2014. Immunohistochemical expression of EGFR was compared between paired primary and recurrent tumor of patients who underwent treatment in the Department of Neurosurgery of Cluj-Napoca Emergency County Hospital and Prof. "Ion Chiricuta" Institute of Oncology. In addition, expression of both immunohistochemical markers was compared with the clinicopathological data of the selected patients, including sex, age, tumour location, treatment (surgical resection,

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radiotherapy, chemotherapy), radiation dose and mean survival.

Tumour samples as well as clinical data were obtained from the Institute database according to protocols approved by the ethical committee and protocols followed the guidelines by the Helsinki Declaration.

Specimens were fixed in 10% buffer formalin and paraffin embedded; histological sections were cut at 5-µm thickness and stained with Hematoxylin and Eosin method.

Immunohistochemistry

Five µm thick sections were performed from each paraffin block. The dewaxing and rehydration of the sections were followed by heat-induced epitope retrieval in citrate buffer pH6 for 30 minutes (Leica Biosystems, Newcastle uponTyne, UK), followed by endogenous peroxidase blocking (3% hydrogen peroxide-5 minutes). Incubation with primary antibody EGFR (Novocastra, Newcastle UponTyne, ready to use, clone EGFR 25, dilution 1:100) had a duration of 30 minutes. To enhance visualisation we used NovoLink Max Polymer Detection System The Leica Biosystems, Newcastle uponTyne, UK and 3,3 - diaminobenzidine as chromogen. Counterstaining was performed with Lille’s hematoxylin. The entire immunohistochemical procedure was performed with Leica Bond Autostainer. Image acquisition and analysis were performed using Nikon Eclipse E 600 microscope and Lucia G software for microscopic image analysis.

The intensity of EGFR staining was assessed on a scale of 0-3+ as follows: 0- no staining, 1+ when a weak cytoplasmatic staining was observed; 2+ when cytoplasm staining is more intense than in 1+, but less than 3+ (Figure 1a), and 3+ when strong cytoplasmatic staining was observed (Figure 1b).

Statistical Analysis

Correlations between EGFR and clinicopathological data were evaluated using Spearman’s rank correlation and Pearson’s chi-square test. P-values of less than 0.05 values were considered statistically significant. Survival data was assessed using Kaplan-Meier curves. All statistical analysis was done using the SPSS 22.0 software (SPSS Inc, Chicago, IL).

Results

Our study included samples from 24 patients composed of 12 males (50%) and 12 females (50%), with ages between 26-78 years (mean 54.33 years). Of the 24 patients, 5 (21%) were treated only by surgery, 3 (12%) received radiotherapy after surgical excision, while 16 patients (67%) underwent surgical resection followed by chemo-irradiation. The median survival was 20.27 months and there were 4 long term survivors who were alive at the time of this study. The interval for tumour recurrence ranged from 1 to 42 months (average time 10.6 months). For the radiotherapy-plus-chemotherapy group the average time was 11 months (p=0.011), as compared with surgery/radiotherapy alone (average time 6.8 months).

We have found EGFR immunopositive staining in 23

patients (96%) with newly diagnosed GBM, including strong reactivity in 15 cases (62.5%), intermediate positivity in 7 cases (29.1%), and weak positivity in 1 case (4.1%). All the recurrent tumours expressed EGFR, with strong reactivity in 9 cases (37.5%), moderate positivity in 10 cases (41.6%) and weak positivity in 5 cases (20.8%). Ten recurrent tumours (42%) had a lower expression than their correspondent pair, 13 tumours (54%) had similar expression, and only one case (2%) had increased expression at recurrence. EGFR expression in newly GBMs was significantly correlated with EGFR expression of relapsed tumours (p=0.023). In addition, newly GBMs with strong EGFR expression (score 3) had a mean relapse-free interval of 11.46 months (p=0.017). By contrast, patients with low/moderate EGFR expression did not show significant correlations with the other clinicopathological parameters. The patient data are shown in Table 1.

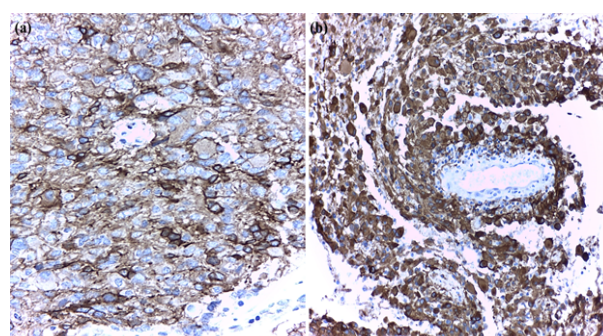


Figure 1. Immunostaining for EGFR in Human Glioblastoma Multiforme. (a) Representative EGFR expression showing moderate (score 2) and (b) strong cytoplasmic staining

Table 1. Patient Data and EGFR Expression

Patient no	Age (years)	Sex	Treatment	EGFR/N	EGFR/R
1	54	M	S+R	++	+
2	71	F	S+C+R	+++	++
3	64	F	S+C+R	-	+++
4	52	M	S+C+R	+++	++
5	26	F	S+C+R	++	++
6	66	M	S+C+R	++	++
7	57	M	S+C+R	+++	+++
8	51	F	S+C+R	+++	++
9	79	F	S	+++	+++
10	38	M	S	++	+
11	38	F	S	+	+
12	43	M	S+C+R	++	+
13	52	M	S+C+R	+++	+++
14	68	F	S	+++	+
15	60	M	S+C+R	++	++
16	78	M	S+R	+++	++
17	58	F	S	++	++
18	40	M	S+C+R	+++	++
19	52	F	S+C+R	+++	++
20	35	M	S+R	+++	+++
21	46	M	S+C+R	+++	+++
22	67	F	S+C+R	+++	+++
23	60	F	S+C+R	+++	+++
24	49	F	S+C+R	+++	+++

N: immunohistochemical expression in newly GBMs, R: immunohistochemical expression in relapsed GBMs, C: chemotherapy, S: surgery, R:radiotherapy

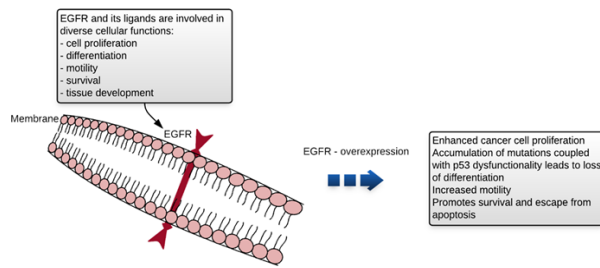


Figure 2. Role of EGFR and its Ligands in Diverse Cellular Functions and EGFR Overexpression in Human Cancer

There was no significant correlation between immunohistochemical expression scores and survival ($p=0.150$).

Discussion

GBMs are highly versatile regarding their cytogenetic and molecular pathways through which they achieve their significant malignant potential. While clinical and biological features of newly GBMs are increasingly well understood, the exact molecular pattern that governs the recurrences is largely inconsistent and inconclusive to promote a certain targeted based medication. In fact, there is no current standard of care for relapsed GBMs.

EGFR, a transmembrane tyrosine kinase receptor, plays an important role in different cancers including GBM. EGFR and its ligands are involved in diverse cellular functions (Figure 2) (Sugawa et al., 1998), and EGFR overexpression was found to contribute to malignant gliomagenesis and poor survival in patients with GBM (Taylor et al., 2012; Hu et al., 2013). In fact, the most common alteration of GBM oncogenes consists of amplification of EGFR gene that results in overexpression of EGFR (Ekstrand et al., 1991; Libermann et al., 1984; Libermann et al., 1985; Wong et al., 1987; Shinojima N et al., 2003). An interesting fact is that in contrast with other tumours that are showing overexpression of EGFR, amplification in GBMs commonly underlines EGFR protein expression (Haas-Kogan et al., 2005).

We assessed the expression of EGFR in 24 paired GBMs by immunohistochemistry, using EGFR.25 clone and we identified overexpression in 96% newly GBMs and in all recurrent pairs. Stark et al analysed EGFR expression in paired GBMs using H11 clone, and he found overexpression in 89% newly GBMs and in only 42% recurrences (Stark et al., 2003). We believe that the high percent of positive tumours in our study is related to EGFR.25, which is able to detect EGFR including EGFRwt and EGFRvIII but also other EGFR type(s) (Shinojima et al., 2003). In addition to the methodology used, discrepancies are likely to appear due to various interpretation of the results (Anagnostou et al., 2010).

In this study we compared EGFR expression in newly GBMs with their relapsed pairs and we have noticed a decreased immunostaining in recurrences ($p=0.023$), similarly with previous studies (Stark et al., 2003; van den Bent et al., 2015). This pattern of expression may be explained by the presence of a distinct subpopulation

of tumoral cells with different genetic profiles (Snuderl et al., 2011; Johnson et al., 2014; van den Bent et al., 2015). However, most of the cases (54%) retained EGFR expression at recurrence and 2% of cases had increased values. These variations suggest GBMs heterogeneity (Del Vecchio et al., 2013; Francis et al., 2014) but also a distinctive pattern of EGFR expression at relapse.

Several studies demonstrated that EGFR overexpression correlates with decreased overall survival (OS) in patients with GBM (Simmons et al., 2001; Shinojima et al., 2003; Pelloski et al., 2007), while others found that the presence of EGFR in GBMs is associated with longer OS (Montano et al., 2001). In our study, EGFR expression had no significant influence on OS ($p=0.150$), consistent with other studies (Stark et al., 2003; Muallaoglu et al., 2014). However, we found that strong EGFR expression in newly GBMs was significant correlated with longer relapse-free interval (11.46 months) compared with cases with low EGFR expression ($p=0.017$). In addition, a benefit of combined therapy was observed in the radiotherapy-plus-chemotherapy group where the average time was 11 months ($p=0.011$), as compared with surgery/radiotherapy alone (average time 6.8 months), similarly with other studies (Pashaki et al., 2014).

In conclusion, our results suggest that EGFR is overexpressed in newly and relapsed GBMs. The discrepancies of EGFR in paired GBMs suggest the heterogeneity of this aggressive tumour but also the unicity of GBM at recurrence. More significant statistics regarding relapsed GBMs would offer a full comprehension of the mechanism that can eventually lead to new anti-tumoral strategies.

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