Original Article

The Relationship of Erythropoietin Receptor Expression and Prognosis in Glioblastoma Multiforme Patients

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ABSTRACT

Background: Glioblastoma multiforme (GBM) is the most common primary brain tumor characterized with poor prognosis and short survival. In addition to the standard treatment protocols, targeted molecular treatment options are under trial. In the recent trials, erythropoietin and erythropoietin receptor were found to be linked with the progression of GBM cells. **Aim:** In this study, we compared the expression of EPOR with survival in GBM patients with mortality. **Materials and Methods:** Twenty-six patients operated for GBM in 2012–2014 were enrolled in this study. Tumor tissues were stained with EPOR, epidermal growth factor receptor, vascular endothelial growth factor, and assigned as (1+), (2+), and (3+) according to their immunohistochemical staining levels. The average postoperative follow-up time was 9.3 months. Kaplan–Meier's survival test and Spearman's correlation test were used in statistical analysis. **Results:** EPOR 1(+) stained group showed a median survival of 8 months (95% confidence interval [CI]: 0.954–15.046). EPOR 2(+) stained group showed a median survival of 6 months (95% CI: 2.901–9.090) EPOR 3(+) stained group showed a median survival of 2 months (95% CI: 0.400–3.600). (Kaplan–Meier $P = 0.002$). **Conclusion:** These results portrayed that EPOR staining levels were inversely proportional with average survival time. In the future, specific inhibitors of this molecule could be used to form a novel treatment option for GBM.

Keywords: *Epidermal growth factor receptor, erythropoietin receptor, glioblastoma multiforme, prognosis, survival, vascular endothelial growth factor*

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Introduction

Erythropoietin multiforme (EPO) is a 165 amino acid long glycoprotein type hormone which is the primary stimulant of erythropoiesis.[1] In recent studies, it was shown that neural stem cells, endothelial cells, and cancer cells also express EPOR.[2] The EPOR regulates the growth and differentiation of normal neural progenitor cells in the central nervous system, and it prevents apoptosis.[3] Clinical studies also suggest that EPO/EPOR signaling is associated with poor survival.[4-6]

In this study, we aimed to investigate the expression of EPOR in patients with GBM and study their relationship with the overall survival (OS).

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Materials and Methods

Patient selection

The primary objective of this study is investigation of the relation with EPOR expression and survival. The classical prognostic factors related to survival are, age at the diagnosis, tumor size, extend of surgical resection, dose of radiation, and adjuvant chemotherapy. All patients of this study were undergone total resection and same adjuvant chemo and radiotherapy. Patients age and tumor size at the

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diagnosis are regarded as factors that can also effect EPOR expression and survival.

Patients and tissues

Twenty-Six patients with GBM (15 male/11 female) were operated in neurosurgery department between 2012 and 2014. The average age of the patients was 59 (range: 28–83). Postoperatively, tissue samples from these 26 patients fixed in 10% formal saline were sent to the histopathology laboratory. The evaluations were performed by two different neuropathologist. Histopathological classification and grading were performed based on the the WHO Central Nervous System Guideline of 2010.

The average follow-up time was 9.3 months (range: 1–32). During this period, 18 patients died (average: 6.8 months, range: 1–16 months), and at the time of this evaluation, 8 patients were still alive. All patients received temozolomide and conventional radiotherapy. Radiotherapy was administered fractionally (a total of 60 Gy was administered over a period of 6 months with daily doses of 2 Gy to the resection cavity and 2 cm of surrounding tissue).

Immunohistochemical staining

Two-micron thick sections from formalin fixed, paraffin-embedded tissue samples were taken into adhesive (polylysine) coated slides. They were put into the oven which was preheated to 70°C for 25 minutes to ensure the complete adhesion of the tissues to the slide. The sticker for the antibody that will be used in the automated BenchMark XT immunohistochemistry device was printed. The slides with the appropriate stickers were put into the device. Deparaffinization was followed by antigen retrieval. At this stage, the slides were left in the EDTA solution for one hour for EPOR (sc-5624 Erythropoietin Receptor [Polyclonal] Santa Cruz), epidermal growth factor receptor (EGFR) (EGFR Antibody (A-10): sc-373746, Santa Cruz), and vascular endothelial growth factor (VEGF) (VEGF Antibody (C-1): sc-7269, Santa Cruz antibodies. After the antigen retrieval, antibodies were added, and antibody titration was performed for 32 minutes. After antibody titration, DAB Detection kit which was compatible with the device was added. The slides were then put in hemotoxilene for 16 minutes and bluing reagent for 4 minutes. The slides were taken out of the device, washed with water and soap, and placed in alcohol. When drying was complete, slides were put in xylene, and they were closed.

Evaluation

Two different, independent pathologists, who were unaware of the patients' clinical situation and other histopathological features, evaluated the slides. Staining of more than 50% of the slides was accepted as positive staining and staining of <50% of the slide was accepted as negative staining. Positive staining slides were then classified as slightly (+), medium (++) and highly (+++) stained categories according to the staining intensity. Membranous and cytoplasmic staining with the EGFR antibody; cytoplasmic staining with EPOR and VEGF were accepted as specific. Nucleolar staining with all three antibodies was accepted as nonspecific [Figures 1 and 2].

Statistical analysis was performed using SPSS version 22.0. (IBM® SPSS Statistics, Armonk, NY, USA). Median, minimum, and maximum values were used as definitive statistical values. The comparison was performed with nonparametric Mann-Whitney U-test. Nonparametric Spearman's correlation test was used to evaluate associations. Survival Analysis was done by "Kaplan–Meier Analysis." Statistical significance was accepted as $P < 0.05$. R values for Spearman's correlation test were discussed based on Spearman's Rho Table.

Results

EPOR, vascular endothelial growth factor, epidermal growth factor receptor immunohistochemical staining

The expression of EGFR was positive in 14 patients; VEGF was positive 21 patients, and EPOR was positive in all 26 patients. Among patients who stained positive with EGFR, 5 were (1+), 4 were (2+), and 5 were (3+). Among patients who stained positive with VEGF, 7 were (1+), 8 were (2+), and 6 were (3+). All of the samples stained positive with EPOR and 13 were (1+), 10 were (2+), and 3 were (3+), respectively. The expression of EGFR, VEGF, and EPOR did not have as statistical significance according to patients' characteristics (age, gender, type of surgical resection, and dose of radiotherapy).

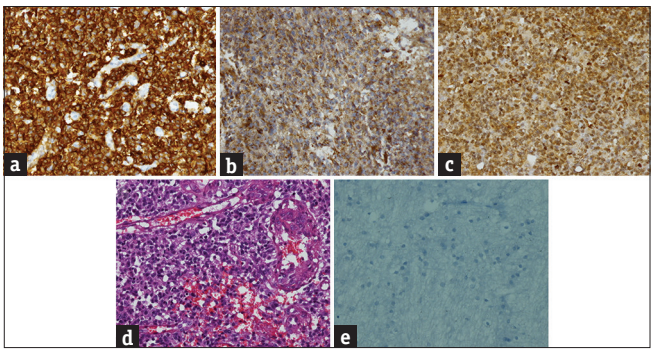

Figure 1: Highly positive immunohistochemical staining patterns seen in the high-grade case (×200) (a) epidermal growth factor receptor, (b) EPOR, (c) vascular endothelial growth factor, (d) HE, (e) negative control

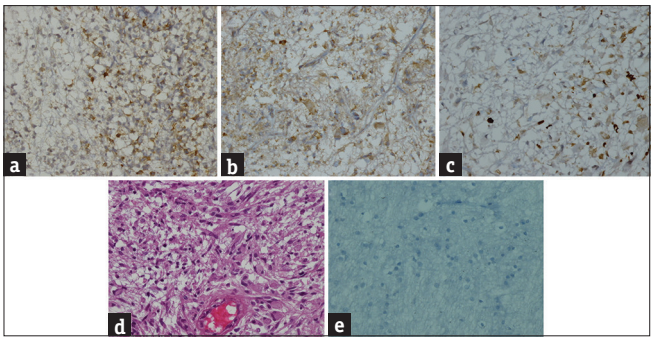

Figure 2: Low-positive ımmunohistochemical staining patterns in a high-grade case (×200) (a) epidermal growth factor receptor, (b) EPOR, (c) vascular endothelial growth factor, (d) HE, (e) negative control

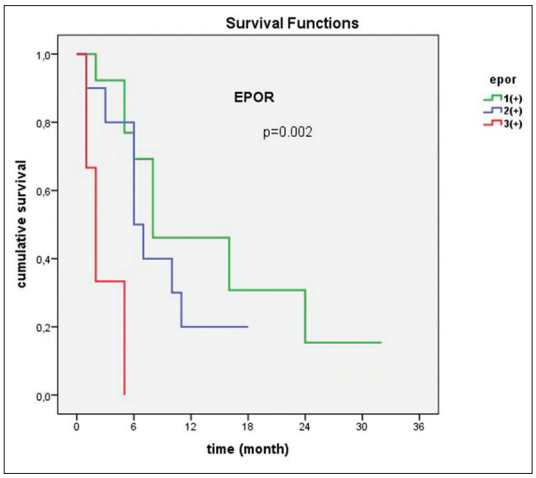

Figure 3: Relationship EPOR and survival

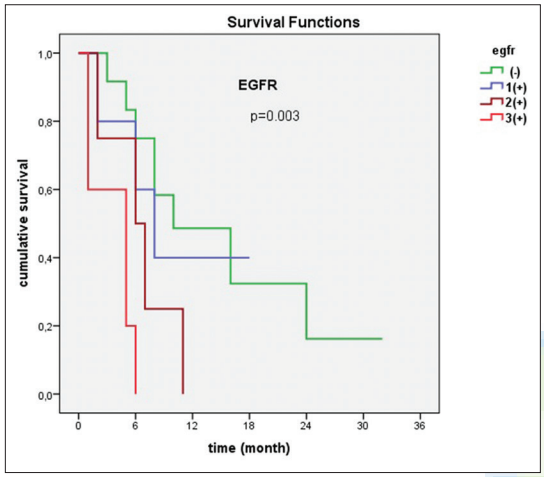

Figure 4: Relationship epidermal growth factor receptor and survival

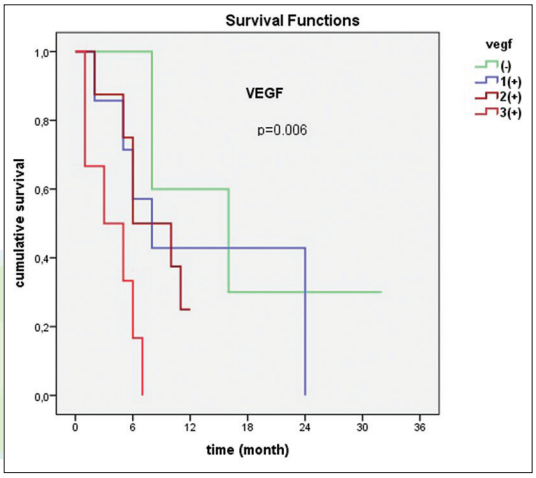

Figure 5: Relationship vascular endothelial growth factor and survival

Analysis of survival

EPOR 1(+) stained group showed a median survival of 8 months (95% confidence interval [CI] 0.954–15.046). EPOR 2(+) stained group showed a median survival of 6 months (95% CI 2.901–9.090). EPOR 3(+) stained group showed a median survival of 2 months (95% CI 0.400–3.600). Level of immunohistochemical (IHC) staining with EPOR and median survival showed statistical significance (Kaplan–Meier $P = 0.002$) [Figure 3].

Among the samples which were not stained positively with EGFR, the median survival was 10 months (95% CI 1.041–18.959). Median survival in EGFR 1(+) stained group was 8 months (95% CI 3.706–12.294). EGFR 2(+) stained group had a median survival of 6 months (95% CI 1.100–10.900). EGFR 3(+) stained group had a median survival of 5 months (95% CI 1.494–8.506). Level of IHC staining with EGFR and median survival showed statistical significance (Kaplan–Meier $P = 0.003$) [Figure 4].

Median survival in patients who were not stained with VEGF was 16 months (95% CI 3.522–28.478). The group who was stained VEGF 1(+) had a median survival of 8 months. (95% CI 2.868–13.132) VEGF 2(+) stained group had a median survival of 6 months (95% CI 1.380–10.620). VEGFR 3(+) stained group had a median survival of 3 months (95% CI 0–7.809). The level of IHC staining with VEGF and median survival showed statistical significance (Kaplan–Meier $P = 0.006$) [Figure 5].

Spearman Correlation Test was used between the level of IHC staining of EPOR, VEGF, and EGFR. There was statistical significant and correlation between EPOR and VEGF ($r = 0.779$, $P = 0.001$) There was a mild and significant correlation between EPOR and EGFR. ($r = 0.465$, $P = 0.017$) There was a mild and significant correlation between EGFR and VEGF. ($r = 0.484$, $P = 0.012$). The positivity of the correlation coefficient in these pathological tissues shows that there is a linear relationship between EPOR, EGFR, and VEGF staining levels. It was shown that with the increase in EPOR levels, EGFR, and VEGF levels were also increased.

Discussion

Since GBM is the most common brain tumor with bad prognosis and short time of survival, a lot of studies are undertaken to discover new treatment protocols. Most of the studies focus on two different molecules (EGFR and VEGF) and their intracellular signal systems. To the best of our knowledge, this is the first study showing that EPO and EPOR expression may have a role in aggressiveness of GBM in respect of prognosis.

The increase in the expression of EPO and EPOR with gene transcription prevents the apoptosis of cerebral endothelial cells causing a neuroprotective effect.[7] The number of studies trying to investigate the expression of EPOR in tumor cells (hepatoma cells, glioma cells, neuroblastoma cells, cervical carcinoma cells, and breast cancer cells) is increasing.[7,8]

It has been shown that EPOR expression is related with increased tumor size,[11] promotion of angiogenesis,[9,10] and prevention of apoptosis.[11] EPOR plays major roles in tumorogenesis, invasion, migration, and apoptosis.[5,12] Nico *et al*. showed that EPO is synthesized in glioma cells and by affecting vascular endothelial cells they promote angiogenesis.[13] Cao *et al*. showed that exogenous EPO treatment lowered EPOR levels and glial stem cell count *in vitro* thus slowed tumor growth *in vivo*.[14] In their study on rodent breast cancer cells, Hardee *et al*. showed an increase in neovascularization in EPO-treated rodents and decrease of neovascularization in EPO antagonist given rodents.[15] Yin *et al*. showed that EPO expression caused increases in antiapoptotic proteins, Bcl-2, and Bcl-xl. They proposed that this increase prolonged the life of tumor cells.[16] Despite this knowledge, there is not enough evidence that EPOR expression increases tumor angiogenesis and proliferation and prevents apoptosis.

The alterations in the EFGR signal pathway, located in chromosome 7q12.2, play a role in *de novo* glioblastoma progression.[17] EGFR is a transmembrane receptor from Tyrosine Kinase (TK) receptor family, which is located in the subset of Erb B receptor family.[18] Intracellular EGFR activation starts with growth factors binding to the extracellular part of EFGR and with the activation of phosphorylated tyrosine kinase intracellularly.[18] With the activation of EGFR, Ras-Mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), signal transducer and activator transcription 3 phospholipase C and SRC/FAK pathways are activated.[17,18] These pathways have functions in cell division and the normal life cycle of the cell.[18,19] Overexpression of EGFR correlates with the grade of glioma. It is seen in 40%–50% of GBM patients and 10%–26% in astrocytoma patients.[20] Therefore, EGFR could potentially be one of the markers that could be used in grading GBMs. EGFR-specific tyrosine kinase inhibitors such as gefitinib and erlotinib have been tried in treatment protocols.[21]

VEGF is a heparin-binding growth factor that is specific to the vascular endothelial cells. VEGF gene has functions in both the physiological and pathological angiogenesis in the brain.[22] VEGF mRNA is found more in the necrotic areas of the brain with GBM. This finding suggests that hypoxia increases angiogenesis in the tumor tissue. Hypoxia increases VEGF ligand in glioblastoma cells and the expression of VEGF receptors in the endothelium of the tumor.[23] The level of VEGF expression is associated with the level of malignancy of the glioma. It was shown that VEGF is expressed 50 times more in glioblastoma.[24] Vascular permeability, ablation of blood–brain barrier, and formation of edema that are seen in glioma are associated with the overexpression of VEGF.[25] Brem *et al*. proposed that endothelial hyperplasia and vascular proliferation were associated with tumor stage in glioma and they showed that high VEGF levels decreased the median survival.[26] Low-molecular weight agents and monoclonal antibodies have been in use to stop this signal pathway. Clinical studies have shown that antiangiogenetic treatment modalities are among the most effective target-specific treatment options in GBM.[27] Bevacizumab is a human monoclonal antibody, and it neutralizes VEGF by binding to it.[28]

In this study, we analyzed the effects of EPOR expression on the survival of GBM patients and its correlation with EFGR and VEGF. In IHC staining, all of the GBM patients expressed EPOR. The patients were evaluated using Kaplan–Meier survival analysis, and the survival times were found to be lower in patients with higher EPOR levels when compared with patients with lower EPOR levels. The median survival in EPOR 1(+) stained patients was 8 months (95% CI 0.954–15.046). The median survival in EPOR 2(+) stained patients was 6 months (95% CI 2.901–9.090) and the median survival in EPOR 3(+) stained patients was 2 months (95% CI 0.400–3.600).(Kaplan–Meier $P = 0.002$) [Graph 2] According to the Cox regression analysis, EPOR expression levels were found to be a worse prognosis indicator independent from OS. ($P = 0.003$) In the Spearman's correlation test performed between EPOR, EGFR, and VEGF according to IHC staining, a strong and statistically significant relationship was found between EPOR and VEGF ($r = 0.779$, $P = 0.001$). A mild and statistically significant relationship was found

between EPOR and EGFR. (r = 0.465, P = 0.017) The positivity of the correlation coefficient between these pathological specimens portrayed the linear relationship between EPOR, EFGR, and VEGF staining levels. At the same time, it was portrayed that when EPOR levels increased EFGR and VEGF levels also increased. Thus, a high level of EPOR expression was associated with worse outcome in GBM patients making EPOR a worse prognosis indicator in GBM patients.

Conclusion

We found that increase in EPOR expression was associated with low median survival in GBM patients. Increased EPOR expression was thought to be associated with tumor growth, angiogenesis, proliferation, and prevention of apoptosis. In the future, specific inhibitors of this molecule could be used to form a novel treatment option for GBM.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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