CHAPTER THIRTEEN

Erythropoietin Promotes Glioblastoma via miR-451 Suppression

Begum Alural[*,†], Zeynep O. Ayyildiz[*,†], Kemal U. Tufekci[*,†], Sermin Genc[*,†], Kursad Genc[†,1]

[*]Izmir International Biomedicine and Genome Institute, Dokuz Eylul University, Izmir, Turkey
[†]Health Science Institute, Dokuz Eylul University, Izmir, Turkey
[1]Corresponding author: e-mail addresses: kkursadgenc@hotmail.com; kemal.genc@deu.edu.tr

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Vitamins and Hormones, Volume 105
ISSN 0083-6729
http://dx.doi.org/10.1016/bs.vh.2017.03.002

Abstract

Erythropoietin (EPO) is an erythropoiesis stimulating growth factor and hormone. EPO has been widely used in the treatment of chronic renal failure, cancer, and chemotherapy-related anemia for three decades. However, many clinical trials showed that EPO treatment may be associated with tumorigenesis and cancer progression. EPO is able to cross blood–brain barriers, and this may lead to an increased possibility of central nervous system tumors such as glioblastoma. Indeed, EPO promotes glioblastoma growth and invasion in animal studies. Additionally, EPO increases glioblastoma cell survival, proliferation, migration, invasion, and chemoresistancy in vitro. However, the exact mechanisms of cancer progression induced by EPO treatment are not fully understood. Posttranscriptional gene regulation through microRNAs may contribute to EPO's cellular and biological effects in tumor progression. Here, we aimed to study whether tumor suppressive microRNA, miR-451, counteracts the positive effects of EPO on U87 human glioblastoma cell line. Migration and invasion were evaluated by scratch assay and transwell invasion assay, respectively. We found that EPO decreased basal miR-451 expression and increased cell proliferation, migration, invasion, and cisplatin chemoresistancy in vitro. miR-451 overexpression by transfection of its mimic significantly reversed these effects. Furthermore, ectopic expression of miR-451 inhibited expression of its own target genes, such as metalloproteinases-2 and -9, which are stimulated by EPO treatment and involved in carcinogenesis processes, especially invasion. These findings suggest that miR-451 mimic delivery may be useful as adjuvant therapy in addition to chemotherapy and anemia treatment by EPO and should be tested in experimental glioblastoma models.

1. INTRODUCTION

Erythropoietin (EPO) is a 34-kDa glycoprotein growth factor and hormone, which controls erythropoiesis through the promotion of proliferation, differentiation, and survival of erythrocytes progenitor cells and survival of mature erythrocytes (Jelkmann, 2013). EPO is initially synthesized in the liver during fetal development, but shortly after birth, production site of EPO subsequently shifts to the kidney. Peritubular fibroblast-like cells in the renal cortex are the major site of EPO production (Suzuki & Yamamoto, 2016). Expression of the EPO and EPO receptor (EPOR) is low in normal adult tissues and mainly induced by hypoxia. Several lines of evidences suggest that the central nervous system (CNS) expresses EPO and EPOR both at the mRNA and protein levels (Maiese, 2016).

EPO was purified from the urine of patients with aplastic anemia, and the therapeutic use of EPO was approved by the US Food and Drug Administration for treatment of anemia in patients with chronic renal failure 30 years

ago (Jelkmann, 2013). EPO is now widely used for the treatment of anemia associated with renal failure, cancer, cancer chemotherapy, prematurity, chronic inflammatory diseases, and human immunodeficiency virus infection (Bennett et al., 2016; Debeljak, Solar, & Sytkowski, 2014). In the last 20 years, many in vitro and in vivo studies showed that EPO has cytoprotective and tissue protective, cell-proliferative, antiapoptotic, antiinflammatory, vascular protective, angiogenic, antiedema, antioxidant, cell migration promoting, neurogenesis stimulation, and metabolism regulation effects (Maiese, 2016). It has in vitro cytoprotective effect against various insults include neurotoxic agents, irradiation, trauma, chemotherapy, hypoxia, ischemia, oxygen glucose deprivation in many cell types (Maiese, 2016). These effects have also been shown in experimental models of various acute CNS injuries, such as stroke and chronic neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (Sargin, Friedrichs, El-Kordi, & Ehrenreich, 2010).

Transport of EPO via the blood–brain barrier to CNS after systemic administration has been observed both in experimental animals and humans at high doses (Brines et al., 2000; Xenocostas et al., 2005). Cerebrospinal fluid concentration of EPO highly increases following intravenous administration (Ehrenreich et al., 2007). In spite of strong preclinical evidences, many clinical studies have failed in small unrandomized and retrospective patient studies. Thus, well-designed, randomized, prospective, placebo-controlled, and larger high quality clinical trials are still needed (Kochanek & Clark, 2016; Pearl, 2014; Sargin et al., 2010). Although several recent studies in stroke, bipolar disorder, schizophrenia patients have not reported negative results, sample sizes are relatively small (Miskowiak, Ehrenreich, Christensen, Kessing, & Vinberg, 2014; Tsai et al., 2015; Wustenberg et al., 2011). EPO is already used routinely in prematurity anemia for a long time (Juul & Pet, 2015). Use of EPO for neuroprotection in very early term infants and neonates with hypoxic-ischemic encephalopathy seems a promising strategy (Juul & Pet, 2015). However, neurodevelopmental deficits and progression of any existing CNS tumor such as pediatric glioblastoma (GB) should be considered. The results of animal studies in the models of age-related chronic neurodegenerative diseases are currently not conclusive. Furthermore, EPO's serious side effects including hypertension, procoagulant, thromboembolic events, and tumor promotion can limit EPO therapy especially in elderly patients (Chong et al., 2013).

GB is the most aggressive and most prevalent primary brain tumor in adults. The World Health Organization classification system groups GB into

four histological grades (Louis et al., 2016). Glioblastoma multiforme, also known as grade IV GB, is the most common and aggressive form of GB (Bush, Chang, & Berger, 2016). The standard therapy for GB is maximal surgical resection followed by radiotherapy and adjuvant temozolomide (TMZ) chemotherapy. In spite of intensive treatment, GB is associated with poor clinical outcome and currently not curable. Recent genome-wide association studies have contributed to the elucidation of the pathogenesis of GB (Bush et al., 2016). Cell surface receptors of growth factors such as epidermal growth factor receptor, signaling pathways, and transcription factors play central roles in the pathobiology of GB. Generally, almost all cancers, in this case GB, are characterized with biological and pathological processes including self-renewal, immortality, unlimited cell proliferation, hypoxia, invasiveness, and metastasis.

Many clinical and experimental studies showed that EPO stimulates tumor growth and progression in many types of cancers (Debeljak et al., 2014). Relatively rare in situ and in vitro studies reported that high EPO and EPOR expression in patients GB tissue samples (Brunotte, Bock, Bruck, Hemmerlein, & Strik, 2011; Mittelbronn et al., 2007; Mohyeldin et al., 2007; Nico et al., 2011; Said et al., 2007). In vitro studies with GB cell lines showed that these cells express EPO and EPOR (Belenkov et al., 2004; Hassouna et al., 2008; Mohyeldin et al., 2007; Peres et al., 2011; Said et al., 2007). In situ studies searched a possible concordance between EPOR expression levels with patient survival and histopathological grade of tumor. Mohyeldin et al. found that expression of EPOR correlated with the stage of the tumor (Mohyeldin et al., 2007). Unexpectedly, an inverse correlation was found between EPOR and GB grade of malignancy (Mittelbronn et al., 2007). However, EPOR expression level is directly associated with survival (Brunotte et al., 2011; Mittelbronn et al., 2007). EPO stimulates cell growth and proliferation in cultured GB cell lines (Hassouna et al., 2008; Yin et al., 2007). EPOR may mediate these effects both in vitro and in vivo experiments (Peres et al., 2015, 2011). EPOR signaling pathways are also involved in responsiveness to EPO (Belenkov et al., 2004; Cao et al., 2010; Kwon et al., 2014). EPO–EPOR signaling may also increase resistance of GB cells to chemotherapy and radiotherapy (Belenkov et al., 2004; Mohyeldin et al., 2007; Peres et al., 2015). Unexpectedly, Hassouna have reported that EPO treatment increased sensitivity to radiation and TMZ (Hassouna et al., 2008). Another interesting finding was that prevention of anemia with EPO-enhanced radiosensitivity of xenografted GB cells (Stuben et al., 2003).

Epigenetic mechanisms and posttranscriptional gene regulation by noncoding RNAs (ncRNAs) can also mediate initiation and progression of GB. MicroRNAs (miRNAs) are short, single-stranded, ncRNAs molecules that regulate gene expression at the posttranscriptional level (Hammond, 2015). miRNAs can control multiple cellular and biological processes including development, proliferation, differentiation, migration, apoptosis, and growth. Association of miRNAs with many human diseases has been extensively studied. Several functional groups of miRNAs such as oncomirs, tumor suppressor miRNAs, angiomirs, and hypoxamirs contribute to GB etiopathogenesis (Costa, Cardoso, Mano, & de Lima, 2015). miR-451 is a widely dysregulated miRNA in several human cancers including GB (Godlewski, Bronisz, Nowicki, Chiocca, & Lawler, 2010; Godlewski, Nowicki, et al., 2010; Pan, Wang, & Wang, 2013; Tian et al., 2012). Different studies shown that miR-451 inhibited cell proliferation, migration, and invasion and induced apoptosis in GB cell lines (Godlewski, Bronisz, et al., 2010; Godlewski, Nowicki, et al., 2010; Nan et al., 2010). Recently, we have determined that EPO downregulates the expression of miR-451 in SH-SY5Y neuroblastoma cell line and biological effects of EPO such as survival, proliferation, and migration depend on miR-451 suppression (Alural et al., 2014).

In the present study we examined the GB promoting effect of EPO in GB cells and the role of miR-451 in this GB promoting effect of EPO.

2. MATERIALS AND METHODS

2.1 Cell Culture and Treatment

Human U87-MG glioblastoma cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biochrom GbmH, Berlin, Germany) and supplemented with heat-inactivated fetal bovine serum (10% v/v), L-glutamine (1% v/v), and penicillin streptomycin (1% v/v). Cells were incubated at 37°C in 5% CO2.

2.2 Presto Blue Assay

Cell viability was assessed by the reducing environment of viable cells which upon entry, converts nonfluorescent resazurin-based solution into highly fluorescents resofurin. Cells were seeded in 96-well plate at a density of 1×10^4 cells per well. Cells were treated with 0–72 h with 0–10 U/mL EPO for basal cell viability. For chemoresistance study, cells were pretreated

with 1 U/mL EPO for 4 h, then incubated with cisplatin at 50 μM for 72 h. At the end of treatment, 10 μL of Presto Blue reagent (Thermo Fisher Scientific, USA) was added to each well containing 100 μL of medium. The plate was then incubated for 30 min at 37°C, and the absorbance of each well were measured on a microplate reader (Varioscan, Thermo, USA) at 590 nm. The relative cell viability was calculated as the percentage of untreated cells.

2.3 BrdU Assay

Cell proliferation of U87-MG glioblastoma cells in response to 72-h treatment of 1 U/mL EPO was measured by BrdU Cell Proliferation Chemiluminescent Assay Kit (Cell Signaling, USA). Cells were seeded in to 96-well plate with a density of 1×10^4 cell per well. These cells were incubated with 20 μL BrdU-labeling solution 16 h prior to the end of 72 h 1 U/mL EPO treatment. Following BrdU incorporation, the culture medium was removed in each well, and cells were fixed with fixing/denaturing solution for 30 min. Subsequently, cells were incubated with BrdU detection antibody for 1 h and HRP-conjugated secondary antibody for 30 min. After several washing steps, 100 μL of substrate solution was added to each well, and the color reaction product was quantified using microplate reader (Varioscan, Thermo, USA) at 425 nm. The relative cell proliferation was calculated as the percentage of untreated cells.

2.4 Western Blotting

The protein samples were extracted from U87-MG cells, and the concentrations were determined using BCA Protein Assay Kit (Sigma-Aldrich, USA). Equal amounts of protein were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and blocked with 5% BSA. After washing three times for 10 min each in PBS (with 5% NP-40), membranes were incubated with EPOR (Abcam), CD131 (Abnova), Ephrin type-B receptor 4 (EphB4) (Abcam), or β-actin antibodies (Abcam) at 1:1000 concentration overnight at 4°C. Membranes were then washed again with PBS (with 5% NP-40), three times for 10 min each, and incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunodetection was performed using enhanced chemiluminescence (Super Signal West Pico, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The density of the protein bands was

analyzed by Image J software (Schneider, Rasband, & Eliceiri, 2012) and normalized to β-actin loading control.

2.5 Immunofluorescent Staining

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and blocked for 30 min in 10% FBS in PBS. Cells were stained with primary antibodies for 2 h at room temperature and the respective Alexafluor-488 or -555 conjugated secondary antibodies (Invitrogen, Germany) for 50 min. Finally, cells were mounted with antifade reagent including 4′,6-diamidino-2-phenylindole (Millipore) for counterstaining and observed with BX61 fluorescent microscope (Olympus, Japan).

2.6 Migration Assay

The U87-MG glioblastoma cells were seeded at a density of 1×10^5 cells/well in a six-well plate and incubated in DMEM containing 10% FBS for 24 h to confluence. A confluent monolayer of each well was scratched with a 1000-μL pipette tip, and cells were washed once with PBS and replaced with fresh growth medium containing 1% FBS, with or without 1 U/mL EPO. Immediately after the scratch (0 h), at 24 and 48 h, wounded areas were marked in each well and images obtained using phase-contrast inverted microscope (CKX41, Olympus) with a 4× magnification. The number of cells that migrated over the margins of the wounds was counted by using Image J software (Schneider et al., 2012).

2.7 Invasion Assay

Thincert cell culture inserts with an 8-μm pore diameter (Greiner Bio-One, Austria) and Matrigel Basement Membrane Matrix (Corning, NY, USA) were used to perform invasion experiments. Prior to experiments, all inserts placed in wells of 24-well plate with sterile forceps. Inserts were coated with matrigel membrane matrix and incubated for 2 h at 37°C. Following incubation, each insert was fixed with 0.1% BSA. U87-MG glioblastoma cells (2×10^4 cells suspended in 1% FBS supplemented media with or without 1 U/mL EPO) were plated in each insert. Media supplemented with 10% FBS was used as chemoattractant in each well, below the insert. The cells were allowed to invade for 24 h at 37°C in a humidified incubator. At the end of the incubation period, cells were stained with Diff-Quick Staining Set (Siemens, Germany), and cells in the upper transfilter were

removed with cotton swabs. The images were obtained using phase-contrast inverted microscope (CKX41, Olympus) with a 20× and 40× magnification. Invasion was quantified by cell counting with Image J software (Schneider et al., 2012) in at least three different fields per membrane.

2.8 Real-Time PCR for miR-451

U87-MG cells were seeded in six-well plate at a density of 1×10^5 cells per well. After treatment with 1 U/mL EPO for 18 h, total RNA was isolated from cells using Qiagen miRNeasy Mini Kit. Quality and quantity of obtained RNAs were measured with the NanoDrop Spectrophotometer (Thermo Scientific, USA). 500 ng RNA was reverse-transcribed using miScript II RT Kit (Qiagen, Valencia, CA, USA). Subsequently, miScript SYBR Green PCR Kit was used to perform quantitative real-time PCR (qRT-PCR) on a Lightcycler 1.5 Real-Time PCR System (Roche Diagnostics, Germany). Both primers (miR-451 and U6) for mature miRNAs were purchased from Qiagen (Valencia, CA, USA). The conditions of qRT-PCR reactions are as follows: 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 30 s. PCR products were evaluated for their specificity by melting curve analysis. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of miR-451 normalized with U6.

2.9 Transfection With miR-451 Mimic

MiR-451 mimic and negative controls were purchased from Qiagen. U87-MG glioblastoma cells were transfected 24 h after seeding in cell culture plates with a density of 1.5×10^3 cells per well in 96-well plate. Transfection of cells with miR-451 mimic and negative control oligomers was performed using the HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. The final concentration of the mimics was 50 nM. After 24 h, media from each well was replaced with DMEM supplemented with 10% FBS or DMEM supplemented with 10% FBS and 1 U/mL EPO and left for 72 h in the humidified incubator.

Transfection efficiency was determined by fluorescence microscope with the use of Cy3-labeled control mimic oligonucleotide.

2.10 Statistical Analysis

Statistical analyses were performed with SPSS 18.0. Results are expressed as mean ± standard error of the mean. Comparison of two groups was analyzed by using Mann–Whitney U test.

3. RESULTS

3.1 EPO Increased Viability of Glioblastoma Cells

To evaluate EPO effect on cell viability, U87-MG cells were stimulated with recombinant human erythropoietin (rhEPO) at different concentrations (0.1–10 U/mL). EPO increased cell viability in a time- and dose-dependent manner as shown by Presto Blue assay (Fig. 1A–C). There was no significant difference between 1 and 10 mL EPO (23% vs 26%) on cell viability at 72 h. Thus, we performed all experiments using only 1 U/mL concentration of EPO.

3.2 miR-451 Overexpression Reversed Cell Viability Promoting Effect of EPO

Using qPCR, we demonstrated that 1 U/mL EPO treatment of U87-MG glioma cells for 18 h significantly decreased basal expression of miR-451 (Fig. 1D). To evaluate a possible functional role of miR-451 on EPO's viability promoting effect U87-MG cells were transfected with miR-451 mimic. High transfection efficiency (over 95%) was confirmed by fluorescent detection of Cy3-tagged negative control miRNA mimic (Fig. 1E). qPCR showed miR-451 mimic robustly upregulated miR-451 level (Fig. 1D). However, contrary to basal miR-451 levels, EPO did not affect ectopic miR-451 expression. We found that the EPO-mediated increase of cell viability was significantly reduced in the mimic transfected cells as compared with cells that were transfected with control mimics (Fig. 1F).

3.3 EPO Promoted Proliferation of Glioblastoma Cells

EPO effect on cell proliferation was determined by measuring the nuclear incorporation of BrdU. 1 U/mL EPO treatment for 72 h led 38% increase in BrdU incorporation suggesting that EPO increased proliferation of U87 cells (Fig. 2A). We further investigated the role of miR-451 on the proliferative effect of EPO in GB cells. We found that the miR-451 mimics transfection significantly reversed the proliferation promoting effect of EPO (Fig. 2A).

3.4 EPO Treatment Increased Resistance to Cisplatin in Glioblastoma Cell Line

We also assessed that EPO treatment has any effect on the response of chemotherapeutic drug cisplatin. Cisplatin application at 50 μM dose

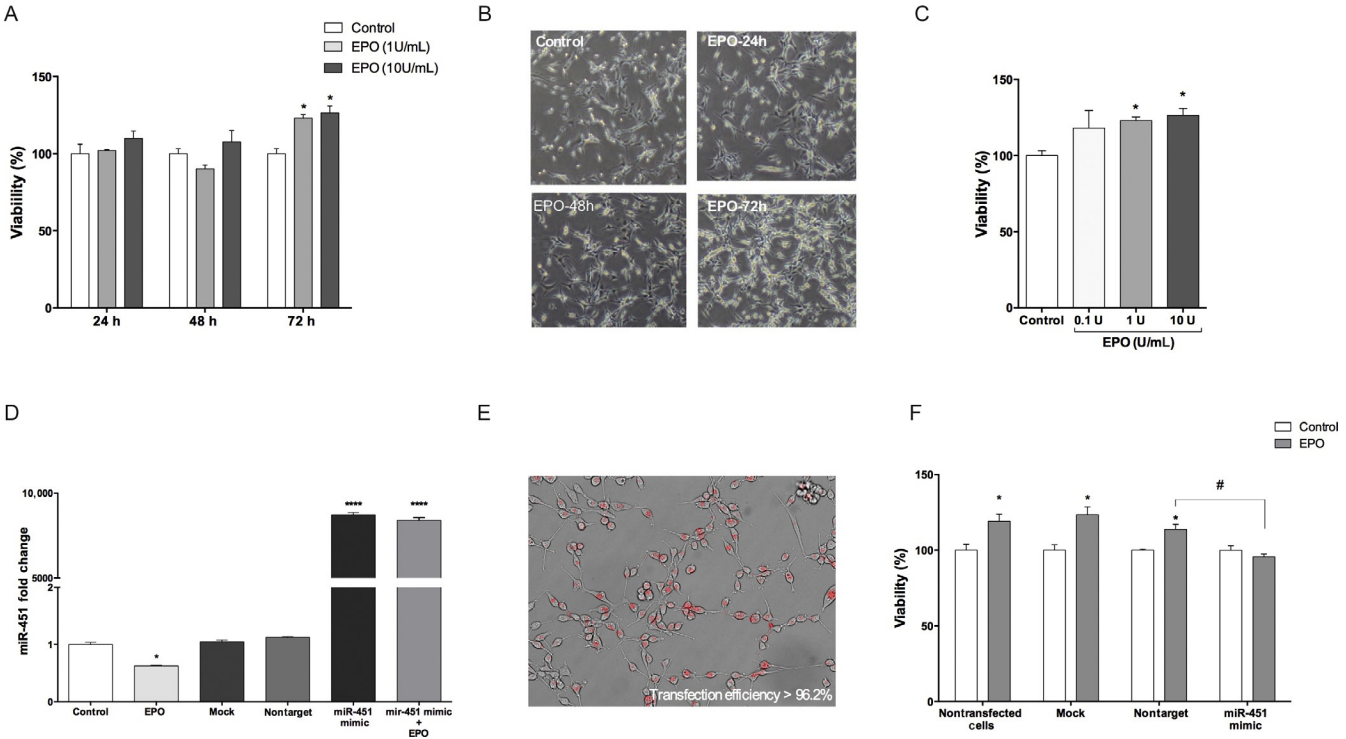

Fig. 1 Erythropoietin increases viability of U87-MG glioma cells. Glioma cells were treated with different concentrations of EPO for 0–72 h, and cell viability was assessed by Presto Blue assay. EPO treatment significantly increased cell viability (A and B) in a time- and (C) dose-dependent fashion. (D) EPO downregulated basal miR-451 expression and miR-451 mimic transfection efficiently induced the miR-451 expression levels in both nontreated (control) and EPO-treated glioma cells. (E) Transfection efficiency was calculated in cells transfected with Cy3-labeled control mimic. (F) Transfection of miR-451 mimic reversed EPO-induced cell viability ($^{*}P < 0.05$ compared to control cells; $^{\#}P < 0.05$ compared to nontarget transfected cells).

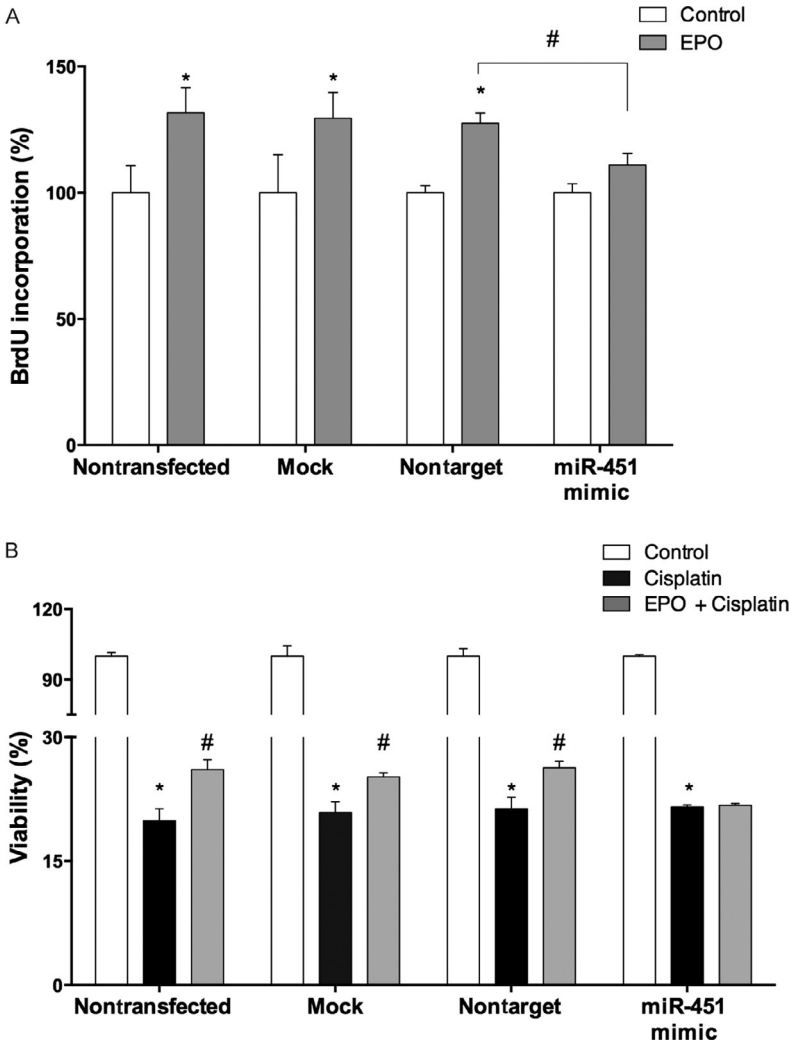

Fig. 2 miR-451 overexpression reversed EPO-induced proliferation and chemoresistance of U87-MG cells. (A) For proliferation analysis, cells were inoculated with 1 U/mL EPO for 72 h, and proliferation was analyzed by BrdU incorporation assay. Transfection of miR-451 mimic reversed EPO-induced cell proliferation. (B) For chemoresistance analysis, cells were pretreated with EPO for 4 h, then cisplatin was added (final concentration: 50 μM). EPO pretreatment increased the cell viability to cisplatin response and miR-451 mimic transfection reversed this effect significantly ($^{*}P < 0.05$ compared to control cells; $^{\#}P < 0.05$ compared to cisplatin-treated cells).

reduces viability to 19%, whereas EPO pretreatment increased the viability to 26% (Fig. 2B). This significant increase in the viability to cisplatin response in EPO pretreated U87-MG cells is a result of EPO-induced chemoresistance to cisplatin. To investigate the effect of miR-451 on EPO-induced chemoresistance to cisplatin, we used miR-451 mimic to simulate overexpression of miR-451. We found that EPO-induced chemoresistance to cisplatin was reversed by miR-451 overexpression in U87-MG cells.

3.5 EPO-Induced Migration of Glioblastoma Cells

Next, we assessed if rhuEPO at the same concentration may have any influence on migration using a scratch assay. EPO treatment increased percentage of migrated cell numbers at 1 U/mL concentration for 48 h (about 80%) (Fig. 3A and B). To investigate the role of miR-451 upregulation on EPO-mediated migration in U87-MG cells, we used mimics to simulate overexpression of miR-451. We found that miR-451 mimic decreased migration of GB cells in both control and EPO-treated groups (Fig. 3A and B).

3.6 Glioblastoma Cells Gain Invasiveness Under Stimulation of EPO Treatment

We evaluated the effects of rhuEPO on cancer cells invasion. Therefore, we examined the effects of rhEPO on the invasion of U87-MG cells across matrigel-coated inserts. 1 U/mL rhEPO treatment for 72 h significantly promoted invasion of U87-MG cells through matrigel (Fig. 3C and D). EPO treatment for 72 h led 137% increase in invaded cells suggesting that EPO increased invasion of U87 cells (Fig. 3C and D). Cell invasion in matrigel was evaluated after transfection with mimics to determine the impact of miR-451- on EPO-mediated invasiveness. miR-451 mimic decreased invasion of GB cells in both control and EPO-treated groups (Fig. 3C).

3.7 miR-451 Overexpression Downregulated the Expressions of Its Own Target Genes

EPO upregulated gene expressions of *BCL-2, MMP-2*, MMP-9, and *VEGF* under basal condition (Fig. 4). Overexpression of miR-451 significantly reduced EPO-upregulated mRNA levels of *BCL-2, MMP-2*, *MMP-9* in U87-MG cells.

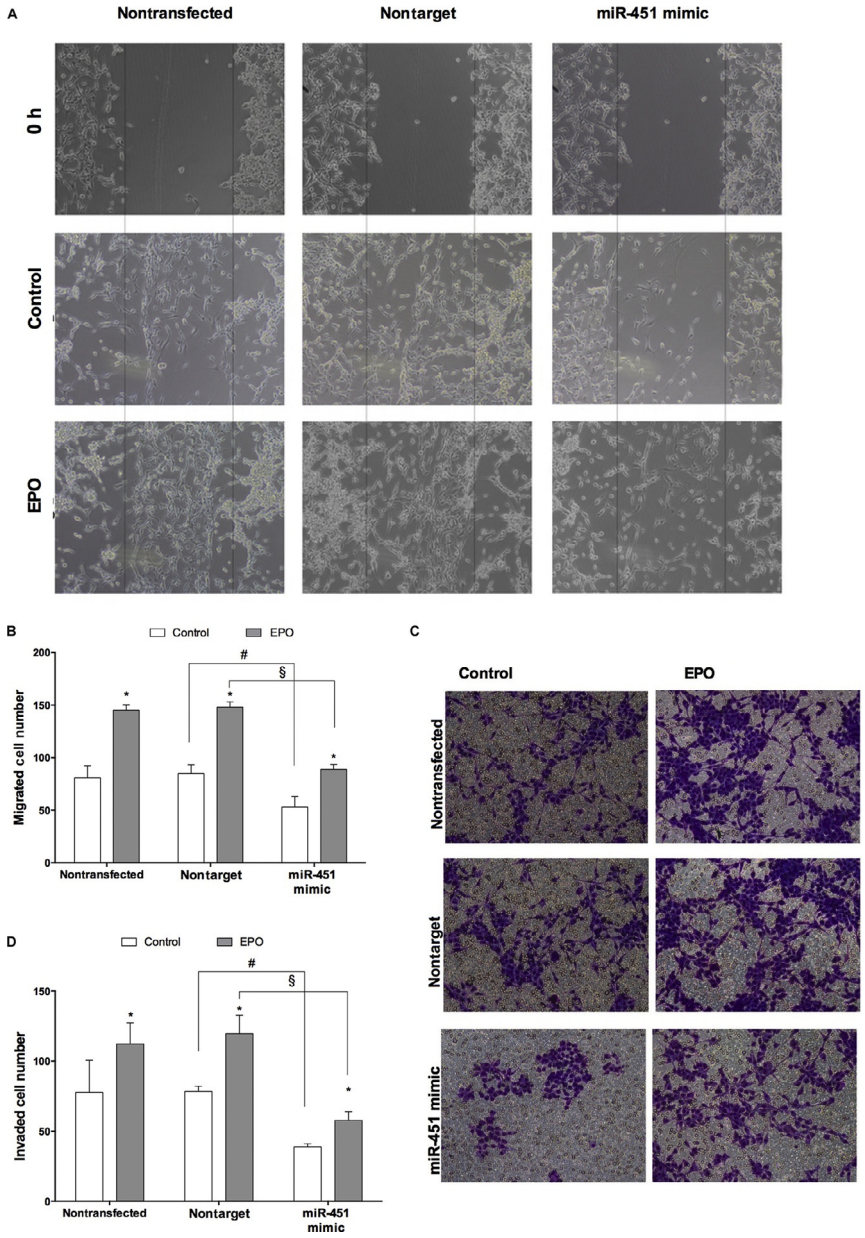

Fig. 3 miR-451 overexpression reversed EPO-induced migration and invasion of U87-MG cells. Treatment with 1 U/mL EPO significantly increased (A and B) migration and (C and D) invasion of U87-MG cells. Transfection with miR-451 mimic decreased migration and invasion of GB cells in both control and EPO-treated conditions ($^{*}P < 0.05$ compared to control; $^{\#}P < 0.05$ compared to untreated nontarget transfected cells and $^{\S}P < 0.05$ compared to EPO-treated nontarget transfected cells).

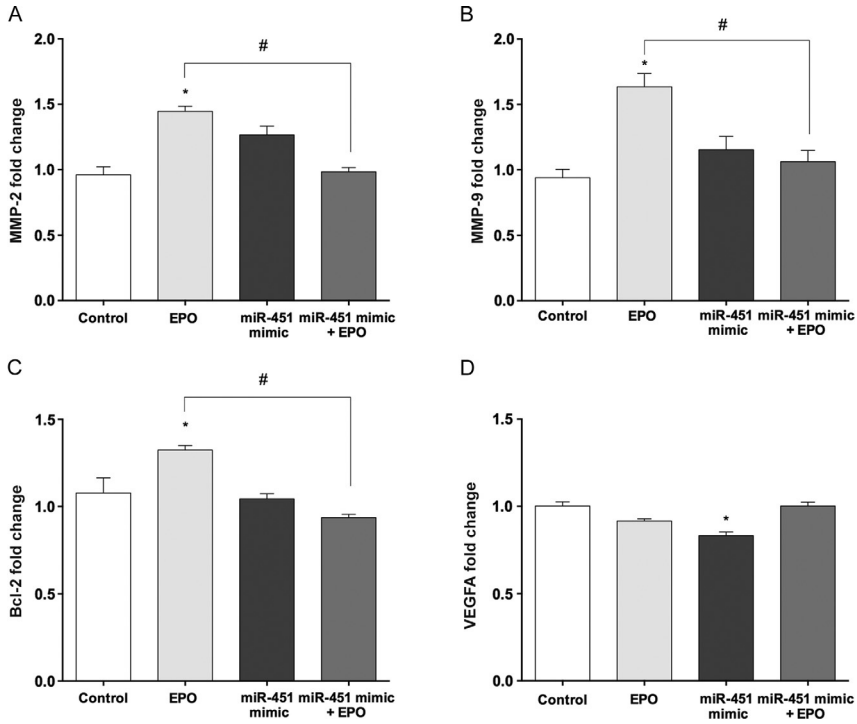

Fig. 4 miR-451 overexpression downregulated target genes induced by EPO. After transfection with miR-451 mimic, cells were treated with 1 U/mL EPO. Expression levels of (A) MMP-2, (B), MMP-9, (C) Bcl-2, and (D) VEGFA were quantified by qRT-PCR (*, #$P < 0.05$).

3.8 U87 Glioblastoma Cells Expressed Different EPORs

Western Blotting and immunofluorescence staining were used to determine the expression of EPORs in U87-MG at the basal level. No primary antibody controls in immunofluorescent staining led to elimination of nonspecific binding of primary antibody to antigen and possible effects of fixation and detergent treatment. We found that U87-MG Glioblastoma cells express three EPO receptors: EPOR, CD131, and EphB4 at the protein levels (Fig. 5).

4. DISCUSSION

The aim of present study was to examine the influences of EPO on cell survival, proliferation, invasiveness, and migration in U87 glioblastoma cell

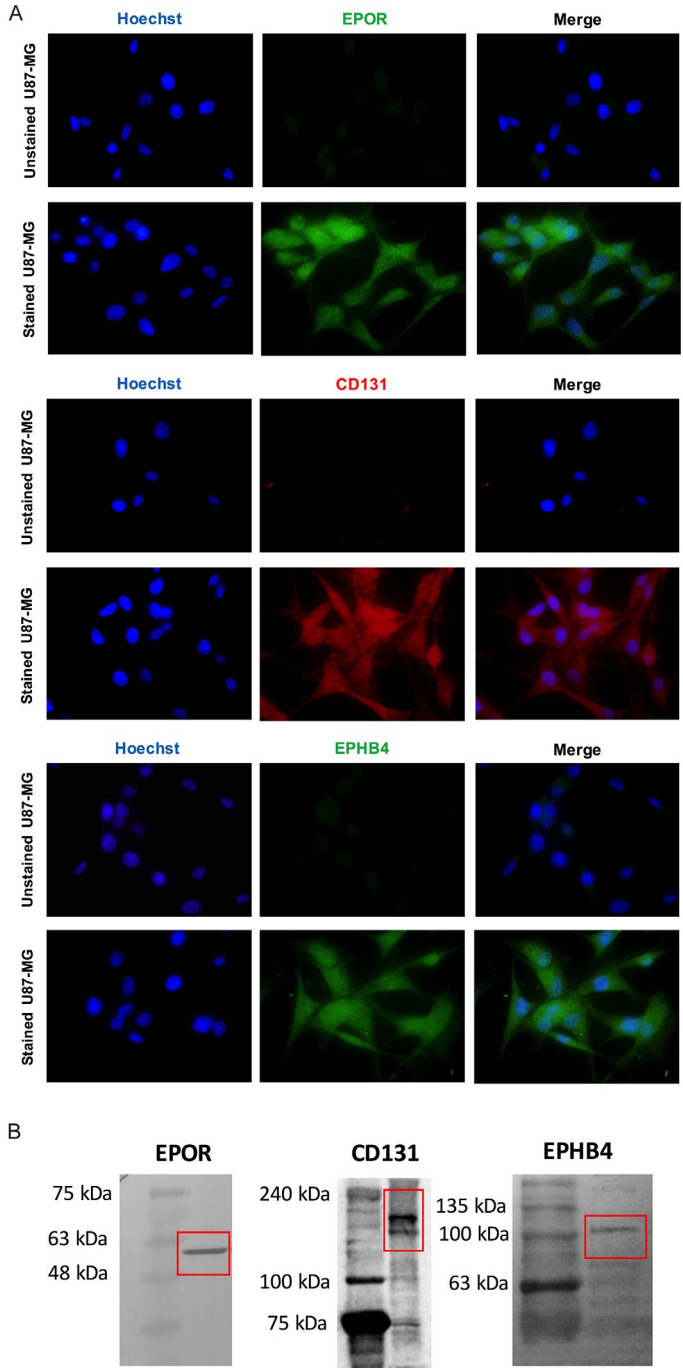

Fig. 5 EPOR, CD131, and Ephrin B4 receptors are present in U87-MG cell line. (A) Cells were plated and stained with related primary antibodies. Cells were visualized under a fluorescence microscope. (B) Protein levels of EPOR, CD131, and Ephrin B4 were analyzed with Western blotting.

line. In addition, we searched whether EPO restores cell survival against cisplatin treatment. Finally, functional experiments were performed to examine the possible reversal effect of miR-451 ectopic expression upon EPO exposure.

We found that 1 U/mL EPO treatment increased basal U87-MG cell survival in a dose- and time-dependent manner. However, EPO significantly increased cell viability only at 72 h. There is no difference between 1 and 10 U/mL doses. We chose 1 U/mL dose for further experiments. Glioblastoma cell proliferation was also increased by EPO. Additionally, our results also demonstrated that EPO rescued GB cells against cisplatin cytotoxicity. The mechanisms of EPO's positive effect on U87-MG cell viability are not fully understood. EPO also protects GB cells from staurosporine, cisplatin, and radiation cytotoxicity, and following EPO treatment, GB cells become more resistance to ionizing radiation and to chemotherapy (Belenkov et al., 2004; Kwon et al., 2014; Mohyeldin et al., 2007). Preincubation of GB cells with EPO resulted in protection against irradiation and TMZ-induced cytotoxicity (Hassouna et al., 2008). rhEPO-induced resistance to ionizing radiation and cisplatin is Janus kinase 2 (JAK2) dependent. Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPK) pathways are responsible for survival effect of EPO against staurosporine cytotoxicity in C6 glioma cells (Kwon et al., 2014). These intracellular signaling pathways also contribute to GB pathogenesis and EPO's signaling (Alifieris & Trafalis, 2015).

We examined the effects of rhEPO on the invasion of U87-MG cells across matrigel-coated inserts. rhuEPO treatment at 1 U/mL concentration for 72 h has significantly promoted invasion of U87-MG cells through matrigel. EPO promotes matrigel invasion of U251 cells dose dependently (Mohyeldin et al., 2007). However, Hassauna et al. did not find any migratory effect with EPO treatment at low concentration (0.3 U/mL) in four GB cell lines including U87 and U251. Functional studies with matrix metalloproteinases-2 (MMP-2) inhibitor confirmed that EPO performs invasion promoting effect in GB cells via MMP-2 upregulation (Mohyeldin et al., 2007). We previously reported EPO-induced expression of migratory and invasiveness genes such as MMP-2, matrix metalloproteinases-9 (MMP-9), and C-X-C chemokine receptor type 4 (CXCR4) in SH-SY5Y neuroblastoma cell line (Alural et al., 2014). GB cells can degrade extracellular matrix via MMPs to gain migration and invasion capacity. Many studies have reported the overexpression of several MMPs, especially MMP-2 and -9 in GB cells compared to their normal cell

counterparts (Paw, Carpenter, Watabe, Debinski, & Lo, 2015). Degradation of the extracellular matrix is also a key event in the tumor angiogenesis allowing the proteolytic destruction of basement membranes by activated endothelial cells and their migration into the tumor tissue for formation of new blood vessels (Onishi, Ichikawa, Kurozumi, & Date, 2011). EPO contributes to GB angiogenesis via a direct effect on endothelial cells, and indirectly by modulating the release of other angiogenic factors such as vascular endothelial growth factor (VEGF) and CXCR4 (Cao et al., 2010; Nico et al., 2011).

MiRNAs and their capacity of simultaneously regulating multiple target genes may play a key role in explaining the complex mechanisms underlying GB formation. They can simultaneously modulate distinct processes such as tumor growth, invasion, angiogenesis, and drug resistance in GB. miRNA-451 is one of the most deregulated miRNAs in GB and has a critical role in GB tumorogenesis and progression. miR-451 maturation process occurs via Dicer-independent, Ago2-mediated noncanonical miRNA biogenesis (Pan et al., 2013). Transcriptional regulation of miR-451 is regulated by GATA-1 and c-myc transcription factors.

In the present study, functional studies performed by ectopic miR-451 expression showed that this tumor suppressor miRNA substantially or completely counteracts promoting effects of EPO on U87-MG cell viability, proliferation, migration, invasion, and chemoresistance to cisplatin. The exact mechanisms of apparent miR-451-mediated reversal of EPO's on tumor cell properties remain to be clarified. However, it seems that miR-451 targets tumor promoting and EPO upregulated survival genes [B-cell lymphoma 2 (Bcl-2), survivin, and Akt)], chemoattractant genes such as CXCR4, tissue remodeling genes that play an important role in tumor invasion (MMP-2 and -9), cell cycle genes [Cyclin D1, cyclin-dependent kinase inhibitor 2D (CDKN2D), and cyclin-dependent kinase inhibitor 2B (CDKN2B)] in various human cancer cell lines including U87-MG (Ehtesham et al., 2013; Godlewski, Bronisz, et al., 2010; Kwon et al., 2014; Nan et al., 2010; Tian et al., 2012; Yin et al., 2015; Zang et al., 2015). c-Myc, transcriptional activator of miR-451, plays a regulator role in cell cycle and also is a target of this miRNA (Pan et al., 2013). We showed that miR-451 inhibits cell survival, proliferative, and migratory effects of EPO in neuroblastoma cell line (Alural et al., 2014). Our ongoing study aims to clarify between EPO/EPOR signaling pathways and miR-451 target genes.

Tumor suppressor miR-451 and growth factor EPO show opposite effects on chemosensitivity and radiosensitivity in different types of cancer

cell lines. EPO induces radio resistance to ionizing radiation and chemoresistance to cisplatin (Belenkov et al., 2004). On the other hand, EPOR knockdown using RNA interference showed that silencing EPOR increases TMZ efficiency and radiosensitivity in GB cell lines (Peres et al., 2015). Transfection of miR-451 mimic sensitizes A549 lung cancer cell line to cisplatin (Bian, Pan, Yang, Wang, & De, 2011). miR-451 also targets MDR-1/p-glycoprotein and increases chemosensitivity to doxorubicin and vinblastine to ovarian and cervical cell lines (Zhu et al., 2008). High levels of miR-451 expression enhanced radio sensitivity of nasopharyngeal carcinoma by directly targeting ras-related protein 14 (RAB 14) (Zang et al., 2015). In our study, miR-451 ectopic expression reversed EPO-induced cisplatin resistance of the U87-MG cell line. The exact mechanisms of chemoresistance and radioresistance of GB cells remain to be clarified. However, our findings may link diverse effects of EPO and miR-451. Multiple signaling pathways, multidrug resistance genes, survival genes, DNA repair mechanisms, and miRNAs may have impact on these processes underlying sensitivity to GB therapy (Koshkin, Chistiakov, & Chekhonin, 2013).

Several studies show that EPOR overexpression in patient GB samples and EPOR silencing experiments may suggest an important role for functional EPOR in GB (Peres et al., 2015, 2011). When EPOR silenced cells were inoculated into nude mice, tumor growth decreased and animal survival increased in preclinical experiments (Peres et al., 2015, 2011). Recently, a modified variant of EPO, carbamylated EPO (cEPO), has been produced and demonstrated that CD131 may mediate cEPO's tissue protective effects (Chen, Yang, & Zhang, 2015). However, any glioma promoting effect of cEPO and CD131 signaling has not been reported to date. In addition to classical EPOR and CD131, EphB4 has been reported as another EPOR and tumor growth and promoting factor in human ovarian and breast cancers (Pradeep et al., 2015). This finding should be verified in GB cell lines and in vivo experimental models. Ephrin and ephrin receptors are involved in glioma and tumor angiogenesis (Ferluga & Debinski, 2014). A possible relationship between these novel noncanonical EPORs and miR-451 should also be considered in GB pathogenesis.

Our present study has some limitations. Comparison of different cell lines would reveal cell-type specific responses (Hong, Chedid, & Kalkanis, 2012). For instance, Hassauna et al. found that low concentration of EPO (0.3 U/mL) results in proliferation in only one (G44 cell line) of four GB cell lines. Second, in vivo xenografted GB model are valuable to dissect EPO–miR-451 link in living organism. Glioma microenvironment consists of various tumor surrounding cells including tumor-associated macrophage,

activated microglia, peripheral immune cells migrated to CNS, vascular cells, glioma, and mesenchymal stem cells (Godlewski, Krichevsky, Johnson, Chiocca, & Bronisz, 2015). Coculture studies using these different types of cells provide a means to study cellular interactions. Intercellular transfer of extracellular vesicles (EVs) loaded with mRNAs, proteins, and miRNAs is also implicated in glioma pathogenesis (Godlewski et al., 2015). miR-451 is enriched in these cargoes and may play an important role in very complex GB microenviroment (Guduric-Fuchs et al., 2012). Indeed, miR-451 contained EVs are transferred from GB cells to microglia and uptaken miR-451 targets c-myc in recipient cells (van der Vos et al., 2016). Such studies highlight the importance of EVs as intercellular mediators. It is very likely many other GB- and cancer-related miRNAs are mediators of EPO's actions. Thus, high-throughput assays and bioinformatics studies should be done before further functional studies.

A growing body of evidence indicates an association between exogenous EPO treatment and tumor progression. However, the mechanisms of tumor growth effect of EPO remain still elusive. In addition to classical EPOR, noncanonical EPORs are also likely mediated to EPO's cell and tissue protective effects and further functional experiments targeting EPORs' are warranted. EPO treatment in cancer patients with anemia and chemotherapy-induced anemia is still a good alternative to blood transfusion. Moreover, the extension of EPO treatment to several neurological diseases and injury is not unlikely in the future and local undesirable effects of EPO on GB may also come up. Long-term EPO administration is a serious problem with regard to tumor progression. Our results have revealed a link between cellular characteristics of GB cells and EPO-mediated inhibition of tumor suppressor miR-451. MiRNA mimic therapy targeting tumor-promoting genes may be a good candidate as an adjuvant therapy in GB.

ACKNOWLEDGMENT

The authors wish to thank Dr. William Gault for his careful and critical reading of the manuscript. This study was supported by Dokuz Eylul University (Grant Number: 2016.KB.SAG.008).

Conflict of interest disclosure: The authors declare no competing financial interests.

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