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ORIGINAL ARTICLE

Metformin plus temozolomide-based chemotherapy as adjuvant treatment for WHO grade III and IV malignant gliomas

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Summary

Purpose: *Glioblastoma multiforme (GBM) remains one of the most devastating diseases known to man and affects more than 17,000 patients in the United States alone every year. This malignancy infiltrates the brain early in its course and makes complete neurosurgical resection almost impossible. Recent years have brought significant advances in tumor biology, including the discovery that many cancers, including gliomas, appear to be supported by cells with stem-like properties. In the current study we have investigated the effects of combining metformin with the standard treatment-of-care, as this drug, already used in the treatment of diabetes mellitus, has shown surprising results in the treatment of breast cancer, being also associated with lower mortality in several other malignancies.*

Methods: *The subjects of the current study were 8 patients with newly diagnosed high-grade gliomas, operated at the Department of Neurosurgery - Clinical University Emergency Hospital, Cluj Napoca. Tumor tissue cultures were established and characterized using immunofluorescence microscopy and PCR analysis and the sensitivity to metformin, epidermal growth factor (EGF) and temozolomide (TMZ) was tested. Microvascular density (MVD) assay was performed on the tumor samples.*

Results: *Seven of the 8 cases had a positive correlation between the number of endothelial cells, the phenotype of isolated tumor cells and the response to adjuvant chemoradiotherapy. The isolated tumor cells had a stem-like behavior, being resistant to conventional drugs. In most cases there was no statistical significant difference between TMZ alone and TMZ plus EGF arms, but there was a important difference between TMZ alone and TMZ plus metformin arms in 6 of the cases.*

Conclusion: *New drugs and targeted molecular therapies are important for future therapeutics, but sometimes we must not exclude drugs already used in the clinic that might have remarkable results. Such is the case of metformin, a drug used for decades in the treatment of type 2 diabetes mellitus that has proven to enhance the effect of TMZ in the treatment of breast cancer and, starting with this paper, of brain cancer.*

Key words: adjuvant chemotherapy, malignant gliomas, metformin, primary tumor cell culture, temozolomide

Introduction

Malignant gliomas are highly infiltrative and lethal cancers of the central nervous system, especially GBM, also known as World Health Organisation (WHO) grade IV astrocytoma. Malignant gliomas also comprise WHO grade III brain tumors, such as anaplastic astrocytoma, mixed anaplastic oligoastrocytoma and anaplastic oligodendroglioma [1]. GBM remains one of the most devastating diseases known to man and affects more than 17,000 patients in the United States alone every year. This malignancy infiltrates the brain early in its course and makes complete neurosurgical resection almost impossible [2]. Postoperative radiotherapy (RT) prolongs survival but yields a median survival of <1 year, and although the most promising early results with adjuvant chemotherapy contained nitrosoureas, no trial proved to significantly prolong survival [3,4].

In 2005, the European Organisation for Research and Treatment of Cancer (EORTC) and the National

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Cancer Institute of Canada (NCIC) published a randomized prospective trial comparing RT alone with RT plus concurrent TMZ followed by 6 months of additional adjuvant TMZ (RT+TMZ) treatment in patients between 18 and 70 years old diagnosed with GBM. The RT+TMZ arm of the trial showed improved median survival from 12.1 to 14.6 months, apart from increasing the percent of living patients at 2 years from 10 to 26% [5]. This is the first modern study that has shown a chemotherapy-related survival benefit in malignant gliomas and was rapidly adopted worldwide as standard therapy for newly diagnosed high-grade gliomas.

Recent years have brought significant advances in tumor biology, including the discovery that many cancers, including gliomas, appear to be supported by cells with stem-like properties [6,7]. Such data in a wide variety of malignancies have demonstrated that only a distinct subpopulation of tumor cells known as cancer stem cells (CSCs) contain the ability to undergo self-renewal and differentiation (properties of normal stem cells) and hence have the ability to initiate tumorigenesis and support ongoing tumor growth. Also it appears that, similar to their normal stem cell counterparts, CSCs have increased resistance to standard cytotoxic therapies and in this way explain the resistance to standard chemoradiotherapy observed by clinicians [8-10]. All these findings have coalesced into the cancer stem cell theory of tumorigenesis, with remarkable implications in our understanding of cancer initiation, progression and patient treatment response.

As the 5-year analysis of the EORTC-NCIC trial demonstrates that new glioma therapies need to be combined with TMZ-based chemotherapy [11], in the current study we have investigated the effects of combining metformin with the standard treatment-of-care, as this drug, already used in the treatment of diabetes mellitus, has shown surprising results in the treatment of breast cancer, being also associated with lower mortality in several other malignancies [12-14].

Methods

Patient population and clinical data acquisition

The subjects of the current study were 8 patients with newly diagnosed high-grade gliomas, operated at the Department of Neurosurgery - Clinical University Emergency Hospital, Cluj Napoca, and for whom snap-frozen samples of the primary tumor available for specific expression analysis have been obtained upon informed consent. Local dissemination was defined as the appearance of an enhanced nodule(s) and/or diffuse enhancement of the leptomeningeal space at sites distant from the primary tumor location on T1-weighted MRI images with contrast enhancement. All the patients were treated according to protocols of the participating institutions (Ion Chiricuta Oncology Institute, Iuliu Hatieganu University of Medicine and Pharmacy and Clinical University Emergency Hospital –all from Cluj Napoca, Romania) and outpatient follow-up was done at 1-2 month intervals. MRI examinations were conducted at least every 2-3 months.

Clinical records, including MRI, were reviewed for each patient. Recorded were patient age at diagnosis, sex, pathological diagnosis, location of the primary tumor, extent of surgical resection, and history of RT and chemotherapy (Table 1). The extent of resection was defined as total (100% resected), subtotal (25-99% resected), partial (5-94% resected) and biopsy. The date of primary diagnosis, the date local or locoregional dis-

Table 1. Patient and disease characteristics and follow-up details. Neurosurgical intervention was subtotal tumor excision. Patients under treatment follow the standard-of-care postoperative therapy of fractionated focal radiotherapy (60 Gy, 30-33 fractions, 1.8-2 Gy/fraction, or equivalent doses/fractionations) and temozolomide at the Department of Radiation Oncology, Ion Chiricuta Oncology Institute, Cluj Napoca, Romania. The microvascular density assay (MDA) value is considered positive with values > 70, according to the Department of Pathology, Iuliu Hatieganu University of Medicine and Pharmacy

Patient number	*Age (years)*	*Sex*	*Clinical diagnosis*	*Histological diagnosis*	*MDA value*	*Follow-up*
1	56	Male	Left intraaxial temporal tumor	Glioblastoma multiforme	61.5	Under treatment
2	53	Female	Right fronto-parietal intraaxial tumor	Oligodendroglioma	78.5	Under treatment
3	67	Male	Right temporo-parietal intraaxial tumor	Glioblastoma multiforme	92	Under treatment
4	44	Male	Left temporal tumor	Glioblastoma multiforme	108.5	Deceased
5	65	Female	Right frontal tumor	Glioblastoma multiforme	84.66	Deceased
6	66	Female	Right parietal tumor	Glioblastoma multiforme	108.5	Under treatment
7	59	Male	Temporo-occipital tumor	Glioblastoma multiforme	130	Deceased
8	48	Male	Right temporal tumor	Anaplastic astrocytoma	123.5	Deceased

semination was detected, final outcome, the date of death or the date of the latest clinical follow-up for living patients were recorded to calculate the time of dissemination and the overall survival.

Reagents

Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from Wörwag Pharma, Bucharest, Romania, diluted in purified water and used at a final concentration of 10 mM diluted in Dulbecco's modified essential medium (DMEM). TMZ was obtained from Schering-Plough, Bruxelles, Belgium, and used at a final concentration of 25 μg/ml. The drug concentrations used in the laboratory equaled the doses used in the clinic.

Ham's F-12 and DMEM used in 1:1 ratio, fetal calf serum (FCS), penicillin and streptomycin were all purchased from Sigma Aldrich, St Louis, MO, USA. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were purchased from Santa Cruz Biotechnology, Santa Cruz, USA. CD31, CD34, CD105 and von Willebrand factor (vWF) monoclonal antibodies were obtained from Research Diagnostics, New Jersey, NJ, USA. CD133, Nanog and Octamer ¾ were purchased from Sigma Aldrich.

Separation and culture of primary glioma cells

Tumor tissue cultures were performed according to the previously described protocol [6]. The fresh tumor tissues removed surgically were washed, minced into 1 mm^3 size with fine scissors, and digested by 0.25% trypsin with 0.02% EDTA at 37° C for 15-20 min; then the digestion was stopped by adding DMEM with 10% FCS. The glioma tissue cells were harvested following filtration and centrifugation at 1000 rpm for 10 min. After the pellet was resuspended with DMEM/F12, all the cells were cultured at a density of 5×10^5/ml. EGF and bFGF were added at a final concentration of 20 ng/ml and cells were cultured at 37° C with 5% CO_2. After 7-10 days of primary culture, cell spheres were observed before being trypsinised and counted using a direct light microscope. The neurospheres were then scattered again by both digestion with 0.25% trypsin and blowing with a small pipette.

Microvascular density assay

Tumors were fixed in phosphate-buffered 4% paraformaldehyde for detection of hypoxia and liquid nitrogen for detection of endothelial cells. CD31, CD34, vWF and CD105 were used as markers of tumor endothelial cells. Immunohistochemistry was carried out by using anti-human rat monoclonal antibody for CD31, CD34 and vWF and the peroxidase technique for anti-CD105 immunology staining. Diaminobenzidine was used as chromogen and hematoxylin was used as counterstaining. Controls included omission of the primary antibody and incubation with blocking peptide before staining. The microvascular density was scored in the invasive front by counting the vessels located within a 1-mm-thick band in the tumor periphery, as described by Miebach et al [15]. Five cross sections were analyzed for each tumor, and images were taken by using an Olympus B×40 microscope and an Olympus C4040 photocamera. All data obtained were analyzed using the Olympus-developed Cell_A® software.

Functional and molecular characterisation

Immunocytochemical staining and reverse transcriptase polymerase chain reaction (RT-PCR) showed that cells isolated from the primary high-grade glioma resection pieces expressed the markers CD133, CD90, CXCR4, Oct-3/4 and GAPDH when compared to placental mesenchymal stem cells, as well as nestin, GFAP and neurofilament protein. The functional and molecular characterisation results were previously published [6].

Cell proliferation assay

Cell survival was assessed using the MTT assay, as previously described [16]. For 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, cells in monolayer culture were irradiated, incubated in DMEM medium supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin before being washed twice with phosphate buffer solution (PBS). Cells were then incubated with trypsin-EDTA, resuspended in culture medium with FCS, counted and plated in 100 μL culture medium at 15×10^3 cells/well in 96-well microtiter plates. After 24 h, cells were washed and treated with metformin before adding TMZ. Absorbance of the MTT was measured at 492 nm using fluorescence microplate reader.

Statistical analysis

Statistical significance values were obtained using a one-way analysis of variance (ANOVA), with 95% confidence (C.I.) level, using GraphPad Prism 5 statistics program (GraphPad Inc, San Diego, CA, USA). Bonferroni's multiple comparison test was considered statistically significant at $p < 0.05$. All experiments were performed in triplicate.

Results

Pathology examination and primary cell culture

Maximal tumor resection was attempted in all patients up to the point that neurological function was not compromised by the extent of the debulking. The resected tumor tissues were examined by experienced pathologists and diagnosed as WHO grade III and IV malignant tumors (Figure 1). The MVD assay evaluated the intensity of the tumor neoangiogenesis and a score of 70 was established as the minimal value for which the assay should be considered significant. Data are shown in Table 1 for all 8 tumors and 7 of the 8 cases had a positive correlation between the number of the endothelial cells, the phenotype of the isolated tumor cells and the response to postoperative chemoradiotherapy. The isolated tumor cells had a stem-like behavior, being resistant to conventional drugs and ionizing radiation, as previously proven [6]. All cell lines had a fibroblast-like appearance and were positive for stem cell specific markers CD133, Nanog and Oct ¾ (Figure 2).

Cell proliferation and adjuvant chemotherapy

In the current experiments, we added both metformin and EGF to TMZ treatment. In most cases there was no statistically significant difference between TMZ alone and TMZ plus EGF arms, but there was an important difference between TMZ alone and TMZ plus metformin arms. Using Bonferroni's multiple comparison test, statistically significant survival data ($p<0.05$) were noticed between TMZ vs. TMZ plus metformin in case 1 (95% C.I. from 0.3770 to 0.5750), case 2 (95% C.I. from 0.1328 to 0.09649), case 3 (95% C.I. from 0.1326 to 0.1483), case 6 (95% C.I. from 0.02170 to 0.1576), case 7 (95% C.I. from 0.07370 to 0.01363) and case 8 (95% C.I. from 0.01163 to 0.05363) (Figure 3).

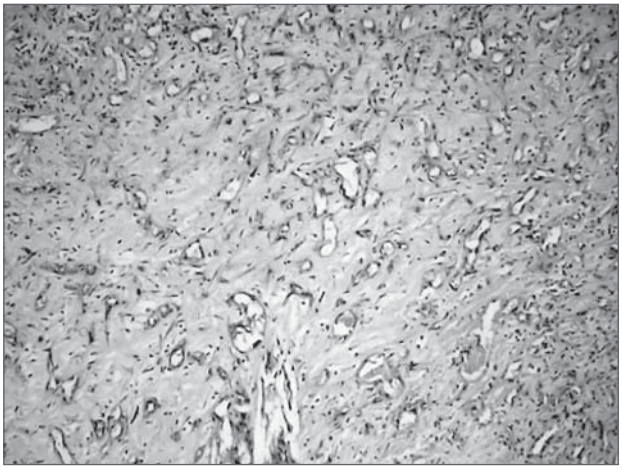

Figure 1. The pathological diagnosis in this case was WHO grade III or IV malignant glioma.

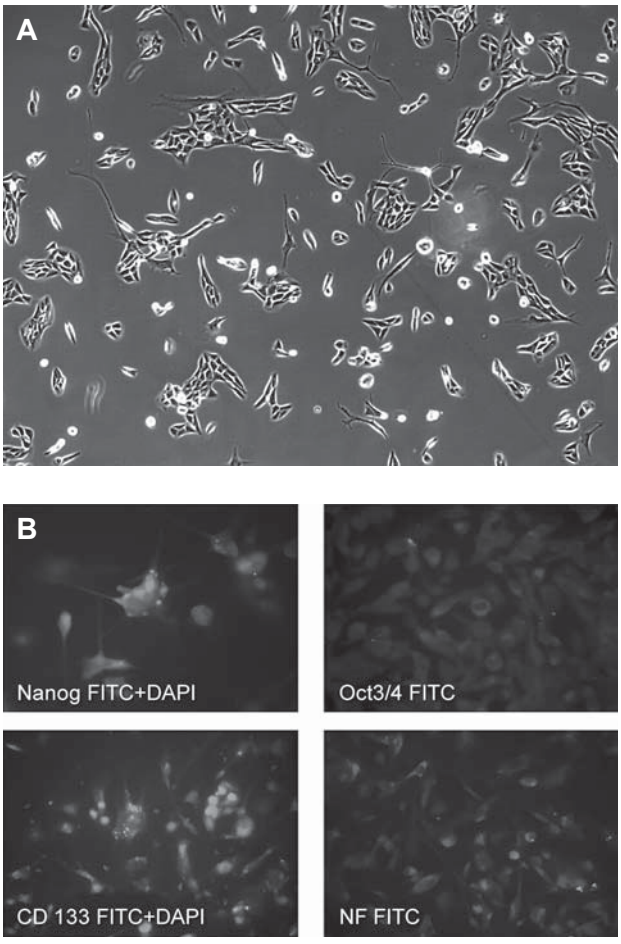

Figure 2. A: After the cultivation of the tumor explants in the described medium, fibroblast-like cells adhered to the bottom of the flask. **B:** After another 5-10 days, the first passage of the cells was carried out. Immunocytochemistry staining of the cell lines proved that the cells were positive for the stem cell-specific markers Nanog, Oct ¾ and CD133, but also for the glial progenitor-specific marker neurofilament.

Discussion

Malignant gliomas are highly infiltrative and lethal cancers of the central nervous system. Focal neurological deficits, symptoms of increased intracranial pressure, epilepsy and cognitive dysfunctions are prominent symptoms in brain cancer patients which may arise in any stage of disease, apart from other reported signs such as fatigue, mood disturbances or anxiety [16]. The highly infiltrative nature of glioma cells often renders a complete surgical removal impossible and inevitably will lead to tumor recurrence, despite state-of-the-art technology in diagnostics such as diffusion-

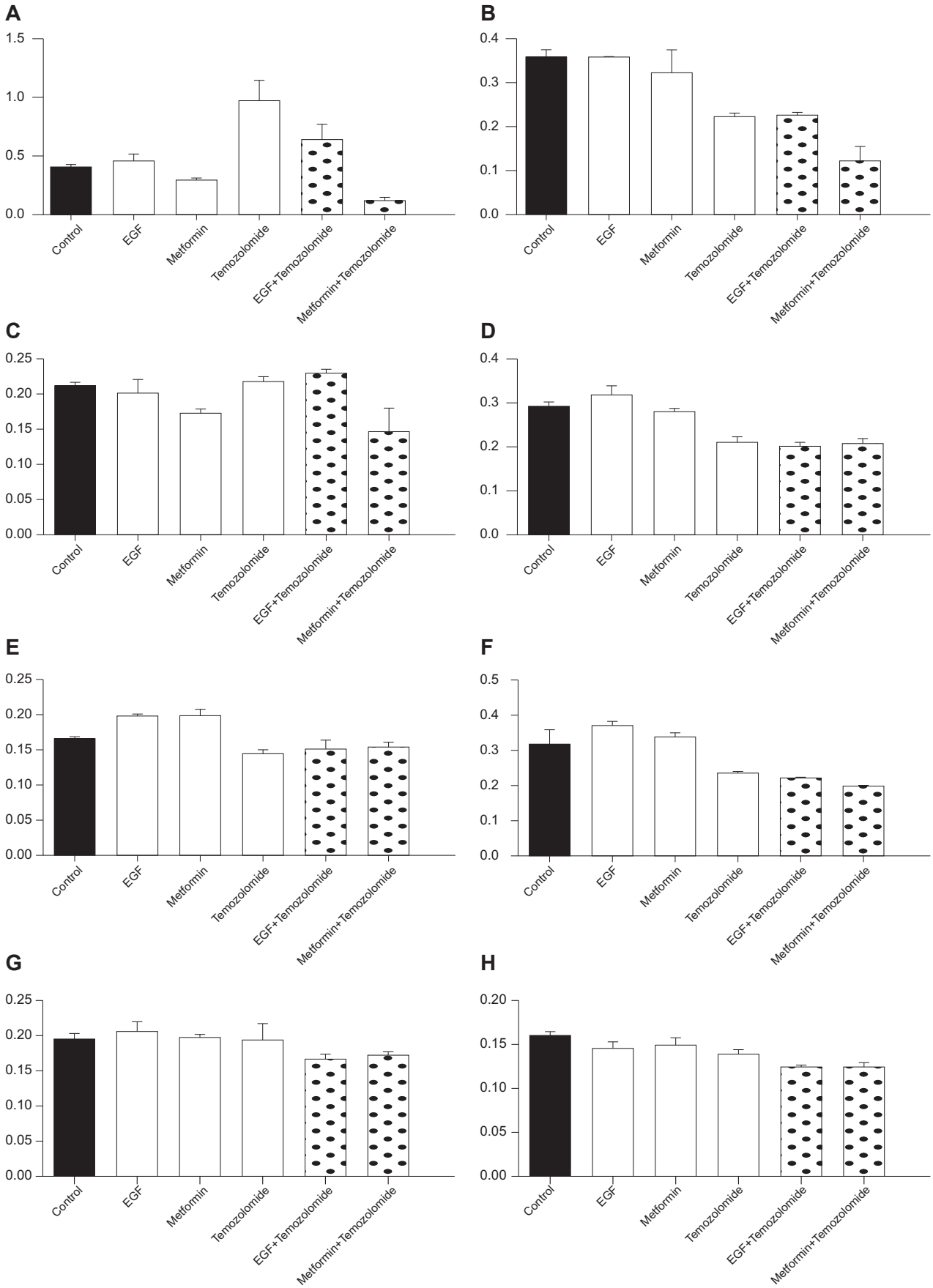

Figure 3. Survival chemotherapy graphics for case 1 (**A**), case 2 (**B**), case 3 (**C**), case 4 (**D**), case 5 (**E**), case 6 (**F**), case 7 (**G**) and case 8 (**H**). The vertical axis represents the optical density and the horizontal axis the various chemotherapy options.

weighted MRI or proton magnetic resonance spectroscopic imaging [17,18].

Long-term survival of patients diagnosed with high-grade gliomas remains poor, with population-based studies estimating that the 3-year survival rate is 5% or less [19,20]. Conventional treatment for newly diagnosed malignant gliomas was traditionally consisted of initial surgical resection followed by fractionated external beam RT, with or without chemotherapy usually using regimens containing alkylating agents. Until recently the benefit of chemotherapy in this setting remained controversial but TMZ, an oral alkylating agent, has proven to be efficient, primarily in the recurrent setting. The large phase III trial conducted by the EORTC and the NCIC demonstrated a significant survival benefit for patients diagnosed with GBM treated with TMZ during chemoradiation and as adjuvant therapy afterwards. Statistically significant improvements were noted in progression-free survival with median 6.9 vs. 5.0 months, overall survival with median 14.6 vs. 12.1 months and a 2-year survival rate of 26 vs. 10%. All results were compared with external beam RT alone [5].

Silencing of the O6-methylguanine (MGMT) gene by promoter methylation has emerged as a potential useful tool to identify patients diagnosed with GBM that can benefited from the addition of TMZ to RT [21]. Such a test is not yet standard-of-care, due to the fact that the study published by Hegi et al. did not take into consideration the relationship between MGMT gene status and TMZ sensitivity and brain cancer stem cells; but this problem seems to be solved by Blough et al. in a very recently published paper in which the authors demonstrated that MGMT status did not predict chemotherapy resistance or sensitivity toTMZ with high precision [22].

Neural tumor-initiating cells, also known as CSCs, display an inherent and extensive tropism to important areas of neurology and oncology and render them as important vectors in future targeted molecular therapies. CSCs migrate long distances within the human brain and specifically enrich the tumor mass of malignant gliomas or brain metastasis. CSCs have been proposed to originate from an early progenitor cell pool located mainly in a 3-5 mm-thick lateral periventricular region of the lateral ventricles, known as the subventricular zone of the brain, apart from the dentate gyrus, subcortical white matter and a subsection of the hippocampal formation - the subgranular layer [23,24]. These regions, known as the stem cell niches, support neuronal stem cells and keep them in an undifferentiated state. The underlying mechanisms of resistance to conventional therapy include cell quiescence, increased ABC-transporter expression, DNA repair and the acquisition of resistance-promoting mutations over time [25]. As it is suspected that the specific niches in the brain also house CSCs, resistant to RT and chemotherapy, and that these tumor-initiating cells need to be eliminated in order to cure brain cancer, Evers et al. performed a retrospective analysis of RT plans of high-grade glioma patients to study the effects of the radiation dose delivered to the periventricular stem cell niches on the progression-free survival of these patients [26]. This study confirmed the CSC hypothesis of neoplasia as the targeted RT of the niches yielded a significant benefit in comparison with standard RT plans.

The CD133+ CSC hypothesis is valid in a wide variety of malignancies, not only in brain tumors. This statement includes breast cancer [27]. Starting from the hypotheses that tumor biology in very similar in both breast and brain cancers and that diabetic patients treated with metformin have a reduced cancer risk, we tested metformin in association with TMZ treatment. This idea has already been tested by Hirsch et al. who have proven that metformin kills CSCs in 4 genetically different types of cancer as a result of the combination of metformin and doxorubicin [28]. These results imply that the combination of metformin and cytotoxic drugs targets both CSCs and non-stem tumor cells and results in reduction of the tumor mass, prolonging remission much more effectively than the use of cytotoxic drugs alone in a xenograft mouse model.

Our results confirm the data obtained by the research team from Harvard Medical School *in vitro* on cell cultures obtained from 8 patients diagnosed with high-grade gliomas. Six of the patients showed a reduction in cell proliferation when metformin was added prior to standard chemotherapy regime using TMZ. The off-patent US Food and Drug Administration-approved drug metformin is the most widely prescribed anti-hyperglycemic agent used worldwide. Metformin is best known as a growth inhibitor which induces glucose stabilization, enhances insulin sensitivity and reduces the circulating insulin levels without associated hypoglycemia. Even if this drug is widely prescribed, its biological and functional activity against oncogenesis is not well understood, but is thought to induce AMP-activated protein kinase activity and inactivates the mammalian target of rapamycin (mTOR), S6 kinase and mRNA translation.

The results presented in the current paper also include data on adding EGF *in vitro*. We have successfully correlated tumor neoangiogenesis with the response to chemotherapy using the MVD assay. This concept is also proven by the results of Wang et al. and Ricci-Vitiani et al. who have demonstrated that CSCs have the ability to differentiate into endothelial cells and thus maintain the stem cell niche in a functional state [29-31]. The

multilineage plasticity and ability to generate endothelial progenitors are new findings in glioma biology, with great impact in the clinic and future therapeutics.

Conclusion

The American Cancer Society estimates over 17,000 deaths annually from primary malignant central nervous system tumors, out of which malignant gliomas account for most of these cases, due to lack of effective therapy. Neurosurgery remains one of the most effective treatments and chemotherapy provides a systemic modality to treat tumors through the central nervous system, nevertheless with efficacy limitations related to drug-delivery issues and inherent resistance.

New drugs and targeted molecular therapies are important for future therapeutics, but sometimes we must not exclude drugs already used in the clinic that might have noticeable results. Such is the case of metformin, a drug used for decades in the treatment of type 2 diabetes mellitus that has proven to enhance the effect of TMZ in the treatment of breast cancer and, starting with this paper, of brain cancer.

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