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Research Article

Chronic Exposure of Glioblastoma Cells to Bevacizumab has no Effect on VEGF-VEGFR2 Signaling and Cell Migration

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Abstract

Background: Bevacizumab (Bev), as very well known antiangiogenic drug targeting vascular endothelial growth factor (VEGF), reduce disease progression in malignant gliomas, but without affecting overall survival. The mechanisms of the resistance to VEGF targeted therapy remains unknown. This study was performed to determine the molecular and phenotypic changes in glioblastoma cells after chronic Bevacizumab exposure *in vitro*.

Methods: Glioblastoma U87 cells were chronically exposed (3 months) to Bevacizumab (0.1 mg/ml) *in vitro* to develop Bevacizumab adapted (Bev-A) cell line. VEGFR expression was determined with flow cytometry. VEGF secretion into the medium was assessed with ELISA kit. Migration was determined using standard *in vitro* assays.

Results: Bev-A cells were found to be unresponsive to Bevacizumab, after three months exposure, and not more migratory than control cells ($p>0.05$). Bevacizumab adapted cells showed high levels of VEGF-A, and low expression levels of VEGF receptor-1 (VEGFR1) and VEGF receptor-2 (VEGFR2), but with no significant difference with control cells.

Conclusion: Our study analyzing the effect on the resistance, by assessing the cells migration on glioma cells treated with Bevacizumab for longer period shows non functional significance of autocrine VEGF signaling. The cells were unresponsive to long term exposure to Bevacizumab. We conclude that in the glioma setting the resistance to Bevacizumab after long term exposure does not include VEGFA-VEGFR2 signaling pathway. The

VEGF signaling pathway for tumor migration and invasion could be more complicated, with open possibility of other VEGF, as VEGFC and VEGFD that could be implicated as alternative pro-angiogenic factors leading to resistance mechanisms from anti-VEGF therapy.

Keywords: Malignant glioma; U87 adapted cells; Bevacizumab resistance; Cell migration

1. Introduction

Angiogenesis is a key event in the process of tumor growth and dissemination. The well-established role of vascular endothelial growth factor (VEGF) in tumor angiogenesis has led to the development of therapeutic strategies that selectively target the VEGF pathway. Anti-angiogenic agents target and inhibits the VEGF itself or the tyrosine kinase part of the VEGF receptors. The anti-angiogenic therapies target tumor endothelial cells, which are genetically stable opposite to cancer cells, which are genetically unstable. Because of that feature, it was thought that the cancer cells are less prone to resistance to anti-angiogenic therapies. Regardless that or the solid tumour type, in some point of time most of the patients treated with anti-angiogenic therapies will develop resistance to it. Mechanisms of the resistance include upregulation of alternative proangiogenic factors, protection of the tumor vasculature either by recruiting proangiogenic proinflammatory cells or by increasing protective pericyte coverage, and accentuated invasiveness of tumor cells into local tissue to co-opt normal vasculature [1-5]. In addition to these proposed mechanisms, currently the focus is increased on the mechanisms of direct action of anti-VEGF agents on cancer cells and tumor adaptation to VEGF inhibition [1, 2].

Several studies, including ours, reported that glioma cells secrete high levels of VEGF and express VEGF receptors. This data support and are in high correlation with the possibility of the escape signal mechanisms because of existence of an autocrine loop [6-10]. In this study, we investigated the molecular and phenotypic changes associated with long time exposure of glioblastoma cells to Bevacizumab *in vitro*.

We found that chronic exposure of glioblastoma cells to Bevacizumab has no impact on migration *in vitro*. The selective inhibition of VEGFR 2, as our laboratory has shown [10] has a clear impact on cell proliferation, apoptosis and cell morphology, but not on cell migration (suppl.1).

2. Materials and Methods

2.1 Cell culture and drugs

The U87 cell line (ATCC, Rockville, USA) was maintained in Eagle's minimal essential medium (EMEM) with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL Penicillin and 100 mg/mL Streptomycin (Lonza, Verviers, Belgium). The U87 cells were exposed to a 0.1 mg/ml dose of Bevacizumab for 3 months *in vitro* to develop the Bev-A cell line. Bevacizumab (Roche, Paris, France) was diluted with culture medium to working concentrations before use. Temozolomide (Mylan, SAS, Saint Priest, France), stock solution was prepared in DMSO at the concentration of 100 mM. SU1498 (EMD Chemicals, San Diego, USA), a selective VEGFR2 tyrosine kinase

inhibitor [11], was prepared as a stock solution of 30 mM in DMSO, then diluted with culture medium to working concentrations before use (all from Sigma Aldrich, Saint-Quentin Fallavier, France).

2.2 Cell growth assay

For cell proliferation assay, parental U87 and Bev-A cells were seeded in 24-well plates (30,000 cells/well) and allowed to attach overnight. The cell viability was then assessed with the MTT assay following Mosmann, 1983 [12], after 24h, 48 and 72h. Briefly, the MTT reagent (thiazolyl blue tetrazolium bromide, Sigma Aldrich, Saint-Quentin Fallavier, France) was added to cells and optical density of the DMSO-dissolved formazan salts was measured after 3 hours incubation. The percentage of surviving cells is expressed as the ratio of optical density of Bev –A cells versus untreated cells (control). For cell sensitivity assay, parental U87 and Bev-A cells were seeded in 24-well plates (30,000 cells/well) and allowed to attach overnight. Cells were then treated for 72 h with different concentrations of Temozolomide (25, 50, 100, 150, 200 µM) in triplicate wells. The cell viability was then assessed with the MTT assay as described above.

2.3 Assessment of VEGFA secretion

VEGFA secretion was assessed with the Quantikine ELISA kit for Human VEGFA (R&D Systems, Abingdon, UK). Parental U87 and Bev-A cells were seeded ($3x10^5$ cells/well) in 24-well plates. The VEGF secretion was assessed, after 48h, in the supernatant following the manufacturer's instructions.

2.4 Assessment of VEGFR1 and VEGFR2 expression

VEGFR1 and VEGFR2 expressions were assessed at the protein level by flow cytometry. Parental U87 and Bev-A cells were seeded ($3x10^5$ cells/well) in 24-well plates, for 24h. In addition, to determine the effect of Bevacizumab on VEGFR-1 and VEGFR-2 expression, 0.1 mg/ml was applied for 24h and then cells were harvested with 1 mM EDTA and adjusted in PBE buffer (PBS containing 0.5% BSA and 2 mM EDTA) to 46106 cells/mL. Phycoerythrin (PE)-conjugated anti VEGFR1 (clone 49560, R&D systems, Minneapolis, USA) and Alexa Fluor 647-conjugated anti VEGFR2 (clone HKDR-1, Biolegend, Saint Quentin Yvelines, France) were added for 30 min. PE-conjugated IgG1 (R&D systems, Minneapolis, USA) and Alexa Fluor 647-conjugated IgG1 (Biolegend, Saint-Quentin-en-Yvelines, France) were used as negative isotype controls. Cells were washed three times and resuspended in PBE buffer and 10,000 events were acquired with a BD LSR II flow cytometer. Results were analyzed using the Cyflogic sofware (Cyflo, Turku, Finland).

2.5 Assessment of cell migration by Scratch-wound migration assays

Confluent U87 and Bev-A cell monolayers in 24-well plates were used. A scratch-wound was made using a 200 µl pipette tip. Wounds were photographed at 0, 1, 6, 9 and 24 hours. Tscratch software (CSELab) using Matlab Compiler was used to measure closure of the wound over time by averaging individual measurement of wound size for six wells at each time point. Two independent experiments with six replicates per experiment were done.

2.6 Statistical analyses

Statistical analyses were carried out using the Student's t-test, with $p<0.05$ as significant value.

3. Results

3.1 Chronic Bevacizumab exposure does not enhance cells growth or sensitivity

To study the effects of chronic Bevacizumab exposure on glioma cells growth characteristics, the cell viability was assessed with the MTT assay, after 24h, 48h and 72h, without and with additional 0.1 mg/ml Bevacizumab. Afterwards the cells were treated with different doses (25, 50, 100, 150, 200 µM) of cytotoxic Temozolomide, for 72h, and the cell viability was than assessed with the MTT assay. There was no difference, neither in growth characteristics or sensitivity to Temozolomide, between Bevacizumab adapted cells and the control (Figure 1a and 1b).

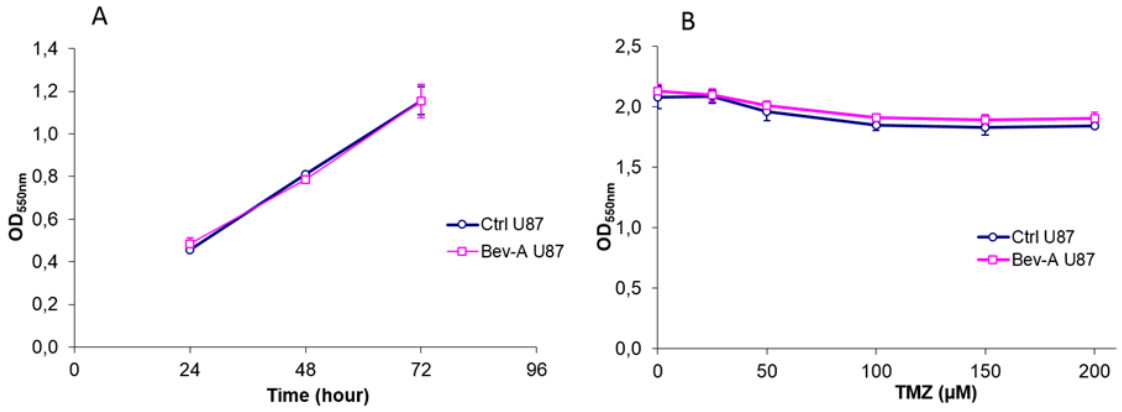

Figure 1: a) Effect of chronical Bevacizumab exposure of U87 cells, on cell proliferation (Figure 1a) and sensitivity (Figure 1b). For cell proliferation assessment, Bevacizumab adapted cells were seeded in 24-well plates (30,000 cells/well), for 24h, 48h and 72h. Addition of 0.1 mg/ml bevacizumab has no additional impact. b) For cell sensitivity assessment, Bevacizumab adapted cells were seeded in 24-well plates (30,000 cells/well), incubated overnight, and treated with different concentrations of temozolomide (25, 50, 100, 150, 200 µM) for 72h. Viability was assessed by a standard MTT assay. Chronical exposure to bevacizumab has no significant impact on cell proliferation or sensitivity to Temozolomide. OD550 – optical density at 550 nm. TMZ – temozolomide.

3.2 Glioma cells chronicaly exposed to Bevacizumab secrete high levels of VEGFA

Bevacizumab adapted cells were seeded ($3x10^5$ cells/well) in 24-well plates and VEGFA concentration in medium was assessed 48 hours later. Glioma cells chronicaly exposed to bevacizumab secrete high levels of VEGFA (Figure 2).

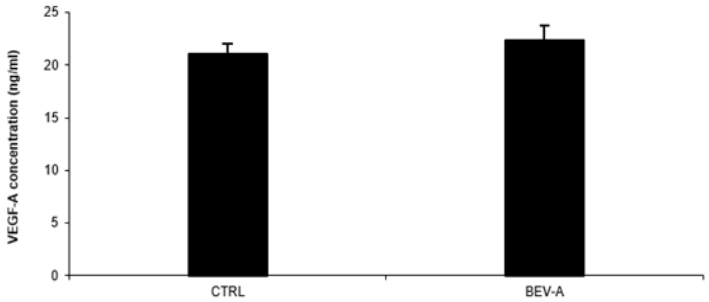

Figure 2: VEGFA concentration of Bevacizumab adapted cell culture vs control in supernatants. Bevacizumab adapted cells were seeded ($3x10^5$ cells/well) in 24-well plates. The VEGFA secretion was assessed after 48h in the supernatants by ELISA. Data are expressed as ng/mL VEGFA (mean 6 standard deviation, n = 3 wells per condition). Glioma cells chronically exposed to Bevacizumab secrete high levels of VEGFA.

3.3 Bevacizumab adapted glioma cells express VEGFR1 and VEGFR2

In addition, FACS analysis of glioma cells chronically exposed to Bevacizumab, showed expression of VEGFR1 and VEGFR2 (Figure 3), with no difference between control cells and Bevacizumab adapted cells.

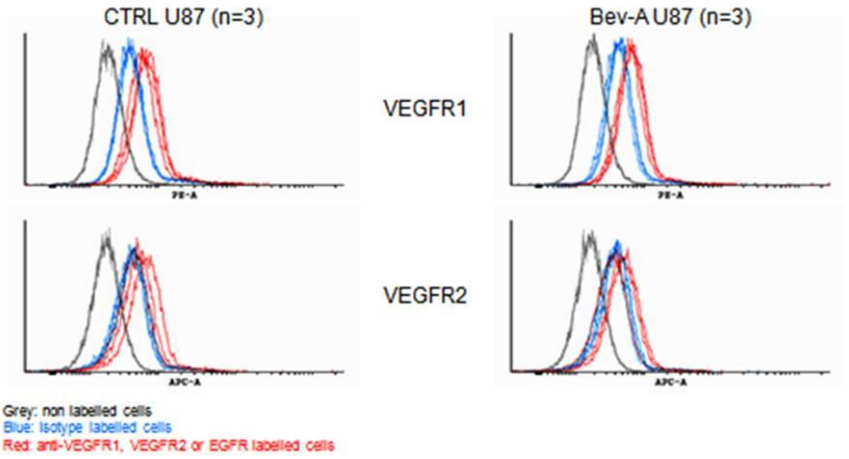

Figure 3: Expression of VEGFR1 and VEGFR2 by Bev-A glioma cells. Bev-A cells were incubated with Phycoerythrin (PE)-conjugated anti VEGFR1 (left panel, red histogram) or with Alexa Fluor 647-conjugated anti VEGFR2 (right panel, red histogram). PE-conjugated IgG1 (left panel, blue histogram) and Alexa Fluor 647-conjugated IgG1 (right panel, blue histogram) were used as negative isotype controls. Representative histograms of duplicate experiments are shown. VEGFR1 and VEGFR2 expression was analysed by flow cytometry. Bevacizumab adapted glioma cells express low levels of VEGFR1 and VEGFR2.

3.4 Chronical glioma cells exposure to Bevacizumab has no impact on cell migration

Parental U87 and Bev-A cell monolayers in 24-well plates were used. The Bev-A cells migrated the same as the control and covered the same area of the scratch (Figure 4a and 4b).

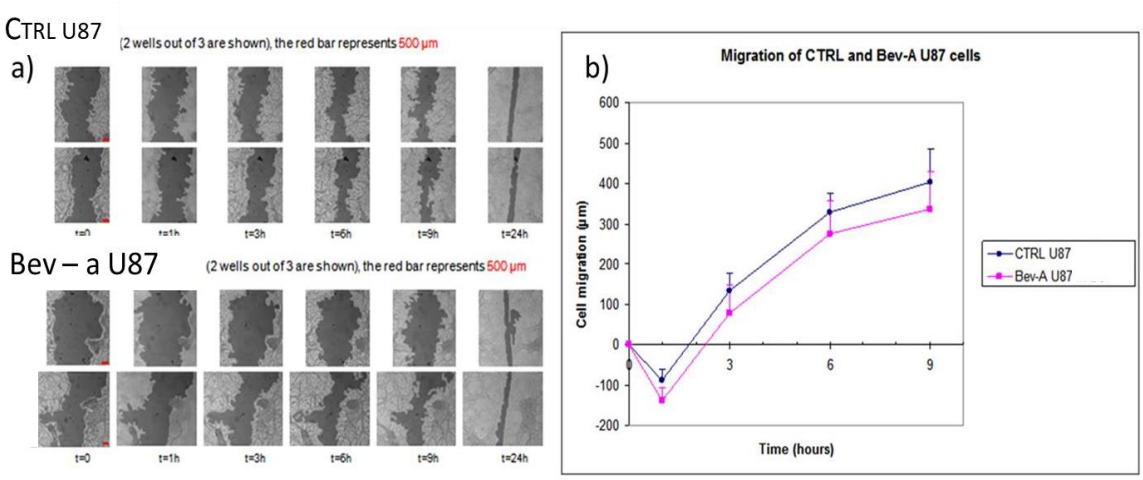

Figure 4: Chronical exposure of glioma cells to Bevacizumab has no impact on cell migration. Using the scratch assay, Bevacizumab adapted cells showed no significant change ($p>0.05$) in migration compared with control cells, covering the same area (Figure 4a) and migrating the same (Figure 4b).

4. Discussion

Bevacizumab, is a monoclonal antibody targeting VEGF. It has been used in the treatment of different solid tumors mostly used in combination with chemotherapy. Bevacizumab has already received regulatory approval and is used in patients tretament suffering with metastatic colorectal cancer (CRC), non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC) and metastatic breast cancer (BC) [13].. High rates of radiological responses and an increase in progression-free survival in both recurrent and newly diagnosed glioblastomas was achieved in clinical studies with Bevacizumab. However, there is no significant impact on overall [14-18]. Factors influencing response and resistance to bevacizumab treatment has opened broad new field of biomarkers research[19]. As one of the highly probable main pathways involved in the development of anti-angiogenic therapy resistance in malignant gliomas has been the VEGFA pathway itself. VEGFA acts by effecting its main receptor, VEGFR2 on endothelial cells. VEGFA is highly expressed and secreted in high levels in the neoangiogenesis sites in solid tumors [20]. Still, the mechanisms of direct action of anti-VEGF agents on cancer cells and tumor adaptation to VEGF inhibition remains unknown, although many preclinical studies and clinical observations has been done [1,21]. Malignant glioma cellssecrete VEGF and express VEGFR, raising the possibility of an autocrine loop signaling as probable escape mechanism that leads to anti-angiogenic resistance [22].

The U87 cell line was selected because the expression of functional VEGFR2 has been reported by different teams, including ours [8,10,11,22,23]. VEGFR2 mediates almost all of the known cellular responses to VEGF [24]. Published data from our laboratory, has shown clear impact of VEGFR2 inhibition on U87 cell line. Namely, we detected changes in glioma cells proliferation, apoptosis and morphology and increased impact on the cells metabolism, assessed by HR MAS spectroscopy, with dramatic increase in lipids, particularly in polyunsaturated fatty acids (PUFAs) and a decrease in glycerophosphocholine (GPC), after treatment with the selective VEGFR2 inhibitor - SU1498 [10]. The U87 cells treated with SU1498, selective VEGFR2 inhibitor, showed a clear impact on

cell proliferation and cell morphology, but no impact on cell migration (suppl.1). There were no changes in glioma cells proliferation, apoptosis, morphology and metabolomics, assessed by HR MAS spectroscopy, after 24h exposure to Bevacizumab [10]. We think that the incomplete neutralization by Bevacizumab and continuously secreted VEGF, at the glycocalyx cells level could be the reason for this kind of U87 glioma cells behavior. The VEGF binds to heparan sulfate, by which is retained on the surface of the cells and in the extracellular matrix [25,26]. Also data shows that the large macromolecules, do not pass through the intact glycocalyx [27]. We propose the same pattern of persistent VEGF/VEGFR2 signaling, as Bevacizumab is a large macromolecule with approximate molecular weight of 149 kDa. Alternatively, for melanoma cells, data has been publish supporting the possibility of the existence of an autocrine loop where cells stimulate their own survival pathways, without the need for exogenous secreted VEGF as the intracrine VEGF/VEGFR2 signalling is smoothly ongoing [28]. This data support the possibility of anintracrine loop protected from antibody blockade as Bevacizumab and accessible to TKI, as SU1498 could be present in U87 glioma cells [29]. The same time exposure of Bevacizumab to glioma U87 cells carrying the IDH1 mutation, assessed by HR MAS can cause changes in glutamine group metabolites and lipids assessed and those could be identified as metabolic markers of Bevacizumab treatment. With this data we have proposed, as the possible mechanism of action of Bevacizumab in IDH1 mutated gliomas, the activation of a functional tricarboxylic acid cycle that runs in reductive manner, and by that we proposed a new target pathway for effective treatment of malignant gliomas [30]. As published by our side, the effect of angiogenic therapy on glioma cells, differs by the type of the angiogenic drug and glioma cell line involved. This study was designed to assess the molecular and phenotypic changes on U87 cells, after chronical Bevacizumab exposure. We treated the U87 cells for 90 days with 0.1 mg/ml bevacizumab. The optimal concentration of Bevacizumab needed to neutralize the secreted VEGFA *in vitro*, was already assessed by our laboratory. Namely, using ELISA kit assay, we assessed the 24h effect of different concentrations of Bevacizumab (1 ng/ml to 0.25 mg/ml) and we showed that concentration above 1 µg/ml completely trapped VEGFA in the supernatant [10].

Here we present that chronical exposure of glioma cells to bevacizumab, has no impact on cell proliferation, sensitivity and migration. Our study shows the absence of effect on migration of chronical Bevacizumab exposure on glioma cells expressing low levels of VEGFR1 and VEGFR2. The effects of angiogenesis inhibitors are transient and the tumors sooner or later develop resistance to the therapy. Kunkel et al demonstrated that glioma xenografts develops infiltrative and invasive growth pattern after 16 days of exposure to anti-VEGF or anti-VEGFR antibodies [31]. Lucio-Eterovic et al reported that glioblastoma cells develops resistance to antiangiogenic treatment by upregulation of other proangiogenic factors, especially the matrix metalloproteinase family members [10]. Liu YX et al, using a quantitative proteomic method has identified more than eighty proteins with significant fold changes, after 48h exposure to Bevacizumab, inducing migration of U87 and U251 cells [32]. However, the exact mechanism and the relative mediators of tumor invasion stay currently unknown. It is an urgent need for the exploration of underlying mechanisms of the drug resistance. The chronical exposure of VEGF inhibitors *in vitro*, has been poorly studied. Usually the Bevacizumab effect on the cells resistance is analyzed by Bevacizumab being applied and studied for the brief time of exposure (48h) in concentrations (> 2,5 mg/mL) that can hardly be reached in patients [10, 32].

Colorectal cell lines (HCT116 and SW480), after three months exposure to bevacizumab, has increased VEGFA secretion and VEGFR1 expression and increased cell migration. After treatment with VEGFR1 inhibitor, SU5416, the cells's migration and invasiveness decreased [33]. The chronic exposure of colorectal cancer cells to Bevacizumab stimulatesalternative pathways that mediate tumour cell migration, throught VEGF-VEGFR1 pathway. VEGFR1 has a very tight ligand binding domain for VEGFA, whereas VEGFR2 has strong tyrosine kinase activity, the two play different roles in angiogenesis through unique molecular mechanisms, but downstream signaling of VEGFR1 has not been fully understood mainly due to the mild biological activity of this receptor in culture [34]. New research data are starting to shed more light on VEGFR1 function and importance.

High expression of VEGF has been correlated with metastasis seeding, and anti-VEGF treatment (bevacizumab) has clinical effects on tumor metastasis. As it looks like, VEGFR1 might be more important than previously thought, especially in the underlying mechanism by which VEGF promotes tumor metastasis. Studies on murine models, shows that anti VEGFR1, but not anti VEGFR2 treatment, can produce inhibitive effects on VEGF-induced tumor metastasis. These findings demonstrate that the VEGF-VEGFR1 signaling pathway is crucial for the development and seeding of tumor metastasis [35]. In the glioma settings the VEGF signaling pathway for tumor migration and invasion could be even more complicated, as data has open the possibility that VEGFC and VEGFD might act as alternative pro-angiogenic factors leading to alterantive and escape mechanisms from anti-VEGF therapy [23]. Also, analysis on LN229 glioma cells, which express VEGFR1, after three weeks bevacizumab treatment (0.25 mg/ml) has shown no significant effect on VEGFR1 mRNA expression in normoxic conditions. In hypoxic conditions the VEGFR1 mRNA was 2.5 fold upregulated, but it had no effect on cells proliferation. Althought in this study, it looks like that the effect of bevacizumab on the expression phenotype of tumor cells might be restricted to certain cancers, VEGFR2 or cell migration was not assessed [36]. More preclinical studies needs to be done though, to have more clear data. Our data shows that U87 glioblastoma cells *in vitro*, secrete high levels of VEGFA and express VEGFR2.

After chronical exposure to Bevacizumab, cells still secrete high levels of VEGFA, and express not only VEGFR2, but also VEGFR1. Nevertheless, autocrine VEGF signaling has non functional significance regarding cell migration, neither after VEGFR2 inhibition (suppl.1), neither after chronic exposure to Bevacizumab.

5. Conclusion

Factors influencing response and resistance to bevacizumab treatment has open new filed by the investigation of biomarkers in order to understand the complexity of the inner cell pathways. The chronical exposure of VEGF inhibitors *in vitro*, has been poorly studied in malignant gliomas setting. We have studied U87 cells adapted to Bevacizumab, after chronical exposure to the drug (0.1 mg/ml, 90 days). Our study analyzing the effect on the resistance, by assessing the cells migration is one of the rarest performed on glioma cells treated with Bevacizumab for such longer time period. Our study shows non functional significance of autocrine VEGFA signaling in glioblastoma cells regarding migration, after chronic exposure to Bevacizumab. The cells were unresponsive to long term exposure to Bevacizumab. Based on our broad research work here we conclude that Bevacizumab is thus less

likely to affect glioma cells due to the therapeutic pressure on the VEGFA autocrine loop regardless the time exposure. In the glioma setting the resistance to Bevacizumab after long term exposure does not include VEGFA-VEGFR2 signaling pathway. The VEGF signaling pathway for tumor migration and invasion could be more complicated, with open possibility of other VEGF, as VEGFC and VEGFD that might act as alternative pro-angiogenic factors leading to alternative resistance mechanisms from anti-VEGF therapy.

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