Cancer- related protein profile of patient-derived and commercial glioblastoma cell lines exposed to Temozolomide

Anna Maria Bielecka Wajdman (bielecka.wajdman@gmail.com)
Medical University of Silesia
Grzegorz Machnik
Medical University of Silesia
Michael Linnebacher
University Medical Center Rostock
Christina Linnebacher
University Medical Center Rostock
Ewa Obuchowicz
Medical University of Silesia

Research Article

Keywords: glioma recurrence, proteome profile of glioma, glioma biomarkers, glioma treatment

Posted Date: April 10th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2782714/v1

Abstract

Purpose

Since recurrence is observed in almost all glioma patients deeper insight into mechanisms responsible for therapy resistance and identification of new biomarkers is urgently required.

In this study were analyzed differences in expression of 84 cancer- related proteins in three GBM cell lines: the commercial T98G cells and two patient-derived cell lines.

Materials and Methods

Influence of temozolomide (TMZ) on changes in proteins expression, cell morphology and migration was investigated. Analyzed lines were characterized by different remarkable plasticity of proteins expression and proteomic alterations induced by TMZ. Among 10 proteins expressed in all lines, 5 (Cathepsin b, FGF, Survivin, AXL, Osteopontin) were modulated by TMZ administration.

Results

As a result of TMZ exposition in both HROG02 and T98G cell lines proteins involved in chemoresistance and invasion (TIE-2, Thrombospondin) were detected. This suggests that TMZ promoted their malignant phenotype even further. In control culture (not subjected to TMZ) of HROG17 cells proteins involved in metabolism were strongly suppressed.

Conclusion

The presented data shed a new light on the immunometabolic profile of glioma proteome and its plasticity in response to Temozolomide interventions. Cathepsin b, FGF, Survivin, AXL and Osteopontin seem to be promising targets for a multimodal treatment that could be applied to inhibit GBM recurrence in the future.

Introduction

GBM is the most agressive form of malignant brain cancer that inevitably recurs despite aggressive standard therapy: consisting of surgical resection, radio- and chemotherapy [1]. Only approximately 7% of patients survive more than 5 years after initial diagnosis. Constant therapeutic failures imply incomplete knowledge about GBM molecular biology and amplify the necessety of new treatment strategies [2].

GBM was the first cancer included in large-scale genetic analysis by The Cancer Genome Atlas project (2008) for which common genetic alterations were identified, but now, to extend this knowledge, recognition of the proteomic changes, as the functional consequences of genomic modifications is very important [3,4,5]. The utility of proteomics for understanding cancer biology and pathogenesis of central nervous system (CNS) diseases is currently one of the most dynamic research areas in medicine.

Although standardi1zation of glioma specific markers is difficult due to heterogeneity, identification of biological signatures by protein profile determination has a high priority since it allows to identify and create libraries of potential clinical biomarkers [6]. Moreover, proteomic analyzes can provide unique information on the basis of monitoring longitudinal response to treatment or enable the design of personalized therapy. All this is necessary because the proposed treatment strategies and options which seemed to be promising such as: oncogenic signaling pathways including RTK/Ras/PI3K p53, pRB signaling pathways, or VEGF-targeting monoclonal antibody Bevacizumab, DNA alkylating agents - lomustine and carmustine implants, and the checkpoint blockade inhibitor thus far have been underwhelming in GBM [7,8,9].

Proteomic researche on cancer cell lines, especially using the directly patient-derived ones is important for identifying biological markers. Although these preclinical models do not perfectly mimic tumor microenvironment, patient-specific alterations at the molecular level are retained to a high degree. Nowadays, proteogenomic and metabolomic studies are conducted on cell line models to: (1) develop and screen for novel anticancer drugs; (2) identify novel mechanisms associated with variation in drug response; (3) understand mechanisms of action for drugs acting directly on tumor cells; (4) predict clinical response to drugs [10, 11]. Despite investigations on mechanisms of GBM invasion in various human samples and preclinical studies, the strong inter/intratumor heterogeneity retains the high demand of searching for novel panels of biomarkers that will better characterize different invasive GBM phenotypes [12].

Since our previous study performed on commercial T98G and primary (HROG02, HROG17) human GBM cell lines showed differences in sensitivity of cells to TMZ, induced by changes of glucose and oxygen concentration in culture medium [13], we decided to investigate if and which differences in proteome profile of analyzed GBM lines could be correlated with this effect. We compared TMZ effects on protein expression in commercial T98G cell line, a GBM lines derived from a patient not treated after resection (HROG02) and a patient with recurrence (HROG17), looking for common or characteristic markers specific for these lines. Moreover, we tried to identify (if existant) a relationship between changes observed in protein expression and the cellular migration as quantifiable feature of cancer malignancy. Finally, for the first time, we carried out microscopic observations of prolonged effect of TMZ on dynamic of confluence by wound healing test, quality and morphology of viable GBM cells in all lines, after removal of dead cells as result of exposition to TMZ. This model was arranged to mimic clinical situation when after TMZ administration resistant cellular subpopulation still exist.

Material And Methods

1. Cell lines and chemicals

T98G (Sigma-Aldrich, St Louis, MO USA) – commercial cell line and routinely used in experimental studies on glioblastoma multiforme; polyploid line established from 61-years old male, that exhibits typical expression of genomic profile and growth factors for mesenchymal GBM cells.

HROG02 (CLS order no. 300931) – primary glioma cell line derived from a patient who underwent two surgical resections for tumor removal (buk resection followed by removal of tumor residues one month later). The development of peritionitis prevented GBM treatment (radio-chemotherapy) and the patient rather had to be treated in department of visceral surgery. Without further GBM treatment patient died 7 months after surgery. HROG02 cell line was molecularly characterized with EGFR amplification, methylated MGMT promotor, *TP53* mutation, and lack of hotspot *IDH 1* or *IDH 2* as well as *B-Raf* mutations.

HROG17 (CLS order no. 300938) – cell line of a glioma relapse derived from a patient who received radiotherapy after the reccurence resection and died 3 months later. HROG17 cell line was molecularly characterized with EGFR amplification, methylated MGMT promotor and lack of hotspot *IDH 1* or *IDH 2* and *B-Raf* mutations.

GBM samples were collected and then cryopreserved as described by Mullins et al. [14].

Medium for cell cultures, gentamicin and fetal bovine serum were purchased from Gibco-BRL (Waltham, Massachusetts, USA). Plasticware was from Falcon (Lexington, TN, USA) and Eppendorf (Hamburg, Germany). TMZ was obtained from Sigma-Aldrich.

Experiments were conducted in **HypoxyLab** (Animalab) to reflect the hypoxic conditions inside the tumor (2.5% oxygen).

2. Protein analysis

For performing protein profile assay, three lines were cultured according to the previously described method [13]. When the confluency reached about 97%, TMZ was added (or not added in control group) to the culture for 24 hrs and GBM lines were incubated in hypoxia conditions (2.5% oxygen). In the next step, cell cultures were rinsed with cold PBS and Ripa buffer with a protein inhibitor was added. Cells from the bottom were scraped off, centrifuged (13000 rpm x15 min) and collected in tubes. The next steps were performed according to the included manual. After signal development, membranes were digitalized using ChemiDoc-It Imaging System (Analytik Jena, Jena, Germany). Thereafter signal-comprised area (dots) were analyzed using ImageJ software. Integrated optical density (IOD) values of a dot that correspond to amount of a protein of interest were measured and calculated while the mean value from dot duplicates was estimated as well. The IOD value was normalized to that of background value for each blot.

3. Separation of alive cells

The LeviCell system utilizes magnetic fields to levitate and separate cells, and does not require dyes, antibodies or specific markers. An inert, paramagnetic compound (Levitation Agent, PN LR-10) was added to the media in which the cells are suspended. Cells are added to a single-use cartridge, which is inserted into the LeviCell instrument where the cells equilibrate in the presence of an externally applied magnetic field. The cells levitate in solution to specific heights determined by the cells' intrinsic properties, including

density and magnetic susceptibility. It can also be used to separate viable cells from dead or dying cells and debris.

Here the LeviCell was used to enrich the viable cell fractions. [total cells loaded] at [X%] viability were mixed with Levitation Agent at a final concentration of 150mM and loaded into the LeviCell cartridge. A total of 220 µL was loaded into the inlet well. Cells were allowed to levitate for 20 minutes, whereby viable cells levitate to the top half of the cartridge chamber, while dead and dying cells sink towards the bottom half. After 20 minutes, the split line is used to set the cutoff for the live cell compartment, and in this case, was set to [insert split line settings]. Viable cells were recovered from the “top” outlet well, and concentration and viability were assessed. The viability was increased to [X%].

4. Wound healing assay.

The wound healing assay is a standard *in vitro* technique for evaluating collective cell migration in two dimensions. Scratch test was performed mechanically with a sterile pipete tip (0.1mm), in the same time of confluency and density for each of line, by the same person.

A sequence of representative images at an interval of 100 minutes in our wound healing assay was carried out in three lines: T98G, HROG02 and HROG17 cultured on p65mm plates using July stage cell analyzer.

5. Microglia phenotype

To determine microglia phenotype, GBM cells (T98G, HROG02, HROG17) were cultured on 35 mm plates with glass bottom (Nunc, Thermo Scientific). After achievieng 70% confluence, cultures were fixed methanol-etanol method. In the next step, to cold PBS buffer (1ml) in the dark was added *Ricinus Communis Aglutynin* fluorescein conjugated and incubated overnight. Next day, plates with cells were washes 3 times with cold PBS and observations were carried out using Olympus BX43 Microscope and 20x magnification lens.

Cell measurements were carried out with the Olympus cellSens Standard program.

Results

1. Protein profile of T98G, HROG02 and HROG17 lines.

Among 84 proteins that were detected, the largest number of proteins (69) was identified in the commercial T98G glioma cell line, then in HROG02 (31 proteins) and the least in relapse HROG17 glioma (21 proteins). In the studied lines the following proteins were mainly expressed (values are expressed in IOD-integrated optical density).

:

– T98G: enolase (**715**), galectin-3 (**580**), FGF basic (460), EGFR (423), p53 (430), survivin (345), cathepsin-5 (305), endoglin (282), progranulin (235), CapG (213), Hif-1α (215)

– HROG02: enolase (450), p53 (290), CapG (306), galectin (270), Hif 1α (210)

– HROG17: thrombospondin (210), kallikrein (163) CD31 (130) angiopoietin-1 (100).

Moreover, in all studied lines, 10 common proteins were found : SPARC, osteopontin, cathepsin b,cathepsin s; HO-1/HMOX1, MMP2, survivin, FGF, Axl, u-plasminogen

2. Protein profile of T98G, HROG02 and HROG17 lines exposed to TMZ

In general, the number of detected proteins in cell lines exposed to TMZ was similar as in the cultures not treated with TMZ. Namely, the number of identified proteins was the biggest in T98G cell line (60), moderate in HROG02 (41) and the least (21) in HROG17 cell line. The same, above mentioned, 10 common proteins were detected in cell lines subjected to TMZ. Moreover, this group of proteins was enriched with TIE-2 and Thrombospondin. In the three lines, expression of some proteins such as FGF, Cathepsin b, Axl, Survivin, and Osteopontin was strongly modulated by TMZ, whereas some proteins did not undergo changes – in T98G cells: MMP-2, VE-cadherin, E-cadherin, GM-CSF, Tenascins; in HROG02 cells: CapG, MMP3, HGFR, Sparc Cathepsin S, H)-HMO-1, EGFR, SPARC; in HROG17 cells: u-plasminogen activator, MUC-1, Cathepsin-s (Fig. 3).

In T98G cell line, exposition to temozolomide remarkably reduced expression of almost all proteins (from 37% for FGF to 90% for IL-2Ra) except for galectin-3, cathepsin-d and vimentin for which expression was increased (from 17% for galectin, 53,8% for cathepsin to even 170% for vimentin). Temozolomid induced production of 2 proteins: TIE-2 and Thrombospondin but also caused complete loss of other proteins: CD-31, Nectin-4, MUC-1, kallikrein, MSP, prostatin, mesothelin, progesterone R 3NR3, cxcl-8 and pdgf.

In HROG02 cell line, exposition to TMZ lead to the appearance of 10 proteins (ICAM-1, PDGFR, TIE-2, IL-6, Snail, p27, ErbB2, CGα/β, Endostatin, Thrombospondin. Moreover, TMZ greatly increased expression of several proteins: p53 (66%), endoglin (125%), Dkk-1 (507%), Axl (235%), FGF (445%), galectin 3 (47%), cathepsin D (73%), vimentin (50%), survivin (46%), serpin (9%) and on the other hand, downregulated expression of a few proteins: enolase-2 (53%), HIF-1α (144%), u-plasminogen (50%), FOX-O1 and MCS-F (88%). The loss of protein expression was not observed.

In the relapse glioma cell line HROG17 TMZ did not induce occurance of new proteins; however it increased the expression of most proteins: kallikrein 5 (30%), IL-2Rα, TIE-2 (92%), cathepsin B (462%), MMP-2 (124%), SPARC (50%), FGF (153%), Survivin (36%), Cathepsin s (26%), HO-1 (56%), AXL (39%), Angiopoietin (15%), HNF3β (25%), VCAM (32%), MUC (15%) and decreased expression of only a few proteins: Thrombospondin, (16%), VE cadherin (84%), Osteopontin (29%), Nectin (66%). Complete loss of protein was not observed (Fig. 1A,B); (Fig. 2A,B,C).

2. Growth and morphology dynamic of separated viable cells after TMZ exposure

Viable cells separated from dead cells after exposition to cytotoxic TMZ concentrations showed differences in the rate of dynamic confluence. We observed that viable cells from two lines: primary HROG02 and commercial T98G line reached confluence faster (after 5 vs. 8 days, respectively). In contrast remaining viable cells from HROG17 did not reach a confluence before day 12. Moreover, HROG 17 cells were characterized by completely different phenotype than other lines. These cells were spindle-shaped with long processes and round, activated cells were not noticed.

Each line reached full confluence at its own pace despite prior TMZ exposure. After 3 weeks, cells of all lines did not detach from the plate bottom as it should be when culture is conducted for long time but they formed multi-layer compact structures. (Fig. 4A,B,C).

3. Migration of cells in wound healing assay

Migration of T98G and HROG02 cells was similar. Time needed for wound healing, considered as an index of cell migration, was about 30 hours for T98G and HROG02 cells and 15 hours for HROG17 cells. In cultures of HROG02 and T98G cell lines, shortly before the wound was completely closed, round cells with the phenotype ofactivated microglia appeared. Such cells were not present in HROG17 cultures (Fig. 5A,B,C).

4. Microglia phenotype

Microscopic observations using agglutinin *Ricinus communis* showed significant differences in morphology of GBM microglia cells especially between relapse HROG17 and HROG02/T98G lines. In the relapse line, as opposed to the other two, cells in the resting phase with numerous processes were visible. In commercial and primary HROG02 line, soma cells were round and presented M1 activated phenotype (area = 3385 and 3776 μm^2 in T98G and HROG02 line, respectively; and 133 μm^2 in HROG17). (Fig. 6).

Discussion

According to some researchers understanding the role of biomarkers in pathogenesis and development of GBM is like a „knocking a Mountain with a Hammer”[15]. However, all scientists and oncologists believe that recognition of mechanisms responsible for GMB invasion/relapse, and distinction of pseudoprogression from effects of local treatment is essential for improving cancer therapy and extending life of patients. In this study, we analized the profile of 84 proteins in three GBM (grade IV) lines: the commercial T98G, the primary HROG02 (from non treated patient) and HROG17 (relapse) exposed to TMZ – the first line alkylating drug used in patients with GBM.

Despite the identical histopathological diagnosis – GBM grade IV (but different status relapse/ treatment) our dot blot analysis indicated a unique protein signature for each line. However, despite strong plasticity in protein expression profiles, among 84 studied proteins we identified 10 proteins in cells with or without exposure to TMZ common in all three studied lines: FGF, Survivin, Axl, Sparc, u-Plasminogen, HO-1, osteopontin, MMP-2, Cathepsin s, Cathepsin b. The main functions of these proteins are related to:

proliferation, migration, pluripotency, immunosupression, intercellular interactions and extracellular matrix degradation. All features emphasize the multifacing of the malignancy features and potentiate the necessity of introducing multidirectional therapy for GBM, focusing on all these features at the same time [16,17,18,19]. However, to this day such a complex therapy scheme has not been practiced. Mostly single target approches are applied. In our model, results of protein profile analysis showe (1) stability of some detected proteins in all lines treated with TMZ or untreated and independent of all other background properties; (2) appearance of new proteins and (3) loss of others. Especially the latter proves dominant phenotypic features of the studied GBM lines and present potential therapy targets in the future.

Results of recent publications indicate that single protein markers have limited reliability in distinguishing tumor subtypes, and that only analysis of (nearly) the entire proteomic profile gives a more comprehensive picture of the protein status [20]. For example, many transcription factors, particularly those involved in the control of cell growth, are unstable proteins and targets for degradation by the ubiquitin-proteasome system [21]. The protein *p53* alone is one of the most commonly dysregulated genes in cancer and the p53-ARF-MDM2 pathway is dysregulated in 84% of patients with GBM and in 94% of GBM cell lines [22]. It seemed therefore that restoring p53 function might be a promissing therapeutic strategy, however previous studies have shown that therapeutic impact is eroded rapidly by the emergence of secondary p53-resistant tumor clones appearing in the face of the selective pressure induced by p53 function restoration [23]. Hence, several recent reports pay attention to the meaning of cross-talk between p53 and Hif 1α as the key players in a complex loop of molecular regulations and common for similar processes such as apoptosis, cell cycle control, response to DNA damage, mitochondrial outer membrane permeabilisation and metabolism [24,25]. In our study, due to the fact that cells of T98G and HROG02 lines are histopathologically confirmed as p53 mutant, we observed that only in these lines expression of p53 and Hif-1α trancription factors was detected and significantly regulated by TMZ. In recurrent GBM we did not observe any protein signal from p53/Hif 1α.

Altered metabolism is one of the prominent malignancy features of GBM that promotes tumor survival and drives recurrence. Our previous study has also shown a strong link between oxygen concentation/glucose availability, TMZ sensitvity and intensification of malignant features in *in vitro* models and relationship between serum glucose and Ki-67 expression in retrospective clinical research [13]. Here, presented findings of proteomic analysis have shown similar expression of proteins involved in metabolism and their modulation by TMZ in models of previously untreated patients (commercial T98G and primary HROG02) and quite different picture in the GBM relapse setting (HRGO17 line). In fact, in the relapsed GBM line all metabolism-related proteins were undetectable except angiopoietin, while in T98G and HROG02 cells strongly expressed enolase-2, p53, Hif 1α and presented with a weaker expression of FOXO-1, MUC1 or carbonate anhydrase.

GBM is also termed „glioneuronal" tumor due to the expression of neuronal antigens on the surface of cells that make up its mass [26]. Neuronal markers are aberrantly expressed in GBM cells. One of them is enolase – an essential enzyme involved in glycolysis-metabolism pathway. It is elevated in many cancers including GBM [27]. This enzyme is physiologically detectable in neurons /neuroendocrine cells. Some

data suggests a role in the adaptation to cellular stress induced by treatment, hypoxia or lack of nutrients [28]. Although enolase expression in patients with GBM is associated with shorter survival, the role of neuronal markers in GBM is still discussed and their relevance seems to not yet have fully been discovered [29]. According to single studies, GBM is able to create electrical synapse with surrounding neurons what helps drive the GBM invasive machinery. Furthemore, GBM cells can be reprogrammed into different subtypes of neurons (with 20–40% neuronal efficacy) while losing tumurogenic capacity at the same time [30]. This kind of ability to transition microglia to neuron-like cells was reported earlier by our team as a mode of action of imipramine (antidepressant). Antidepressants are often prescribed for GBM patients, not only in case of depression but also because therapy side effects such as neuropathic pain or neurological disturbances or as results of cancer progress. However, the role of antidepressants in GBM therapy is poorly understood. Conversion of glioma cells to neuron-like cells, may therefore represent a novel therapeutic strategy and may open a new chapter in treatment of this highly devastating cancer [31,32].

Loss or down-regulation of markers as we observed in a case of enolase expression in relapsed GBM (HROG17 line), may also be a result of previous treatment. Radiation – element of standard therapy induces some paradoxal effects on a celllular level such as upregulation of the hypoxia mediated angiogenesis or rapid shift in GBM subtype from proneural to mesenchymal as early as 6 hours after the procedure [33]. This dynamic proces is characteristic for GBM progression leading to chemio-radioresistance and glioma stem cell (GSCs) stimulation which can explain this divergent protein profile of relapsed GBM in comparison to primary tumors.Enolase as a multifunctional protein appears to be therefore important in maintaining the malignant potential of the GBM also for other reasons. According to some studies, enolase is a critical element in adaptation to cellular stress and (inter) acts with cathepsins (cathepsins cleave the C-terminal dipeptide of γ-enolase). It thus may have an undening role in tumor immunology making a bridge between GBM metabolism and immunology pathways [34]. In consequence, this immunometabolic profile of GBM is supported by immunosupressive tumor microenvironment and is additionally regulated by altered tumor metabolism, creating an immunologically „cold tumor”. Brain metastases and GBM relapses generate changes in brain tissue remodelling that triggers inflammatory response. Which in turn commands and stimulates GBM cell invasiveness especially cell migration and proliferation [35].

Osteopontin, u-plasminogen, HO1-HMOX1, cathepsins and MMP were detected as the common proteins in three analyzed GBM cell lines both in the group of „control conditions” and the group after „TMZ exposition”. In consequence, their constant expression (independent of exposition to TMZ) can generate the potential for degrading the extracellular matrix (especially cathepsins, MMP) and favors recurrence. However „ top”proteins, dominant in the relapsed HROG17 GBM profile seem to place a dot above „I” and decide about invasiveness power (Kallikrein 5 and Thrombospondin). Kallikrein 5 belongs to the serine proteases family and in other experimental models was shown to accelerate migration and increase invasion of cancer cells without affecting the proliferation capacity [36]. Moreover, it has the ability to cleave extracellular matrix (ECM) (collagen type I, II, III, and IV, fibronectin, and laminin) and adhesion molecules (fibrinogen and vitronectin). This is critical for modulating tumor invasion and metastasis [37].

In turn, numerous studies on several cancer types, including GBM, emphasize thrombospondin properties for modulation of immune responses as well as GBM vascularisation. Moreover, this multifunctional glycoprotein has a close association with activation of TGF-β, orchestrates APCs (antygen presenting cells) and, also important, initiates microtube formation thus promoting cytoplasmic extensions of glioma cells, necessary for cell communication but in effect promoting tumor invasion[38].

Up-regulation of above mentioned proteins in proteomic signatures probably goes hand in hand with our microscopic observations. In the test of separating viable from dead cells after exposition to TMZ, the results of the LeviCell system also showed significant differences in morphology and dynamic growth of GBM cells originated from the three studied lines. In relapse GBM cells we observed dominance of the phenotype „go" manifested by a weaker proliferation index, strong plasticity of cell morphology and significant elongation of soma cells in search of cell-cell contact. T98G and HROG02 cells reached confluence twice as fast (full confluence was detected on the 5th day) what indicates the dominance of the „growth"phenotype.

In the wound healing test that can mimic situation taking place after tumor resection and around resection margine, HROG17 (relapse) GBM cell line needed much less time (15 hours) to heal the wound than the HROG02 or T98G cell line (about 30 hours). Cells of relapse HROG17 were also characterized by long soma and more morphological plasticity than cells of T98G and HROG02. It was also interesting that in the commercial T98G and HROG02 lines we detected predominance of microglia cells with active M1 proinflammatory phenotype (round cells with average surface area = 3385 and 3776 μm^2, respectively) and the opposite in HROG17 relapse: resting M2 antiinflammatory form of microglia (ameboid, with average surface area = 133 μm^2).

Since response to CNS damage or GBM surgery is reactive gliosis, angiogenesis and inflammation, the explanation of the observed differences between the analyzed three GBM lines can also concern microglia and its critical role in gliomagenesis and recurrence. Resident microglia and macrophages known as tumor-associated macrophages (TAMs) account for 30–50% of the tumor mass and with parallel low infiltration of functional T and NK cells build an immunosupressive GBM microenvironment [39]. Tumor resection stimulates microglia activation characterized by changes in morphology, polarization (M1, M2), gene expression, proliferation, phagocytic capacity, and migration towards the inflicted injury [40]. Resection trigers also immediate and prolonged effects on the cytokine expression profile. Although microglia cells are executors of the innate immune response and also specialists in sensing/eliminating abnormal (cancer) cells, through flexy changes of their phenotype they become, as subject of signals from glioma cells remained after resection, tumor-promoting cells. Therefore, it is suggested that glioma can hijack the microglial immune response to promote tumor growth and on this basis an idea of depletion of microglia cells in experimental models was born [41]. Results of these experiments showed that depletion of microglia cells induced by PLX5622 (highly selective CSF1R inhibitor capable of brain penetration after oral administration) reduced glioma growth and invasion *in vivo* and indicated that this strategy can turn out an important target for non-invasive therapy and reverse pro-tumorogenic microglia activation [42].

Osteopontin (OPN) seems to also be an attractive target for immunomodulation of microglia. In our analysis this protein was detected in all studied GBM lines [43]. On the basis of the Human Protein Atlas it is known that the highest expression in 17 of the analyzed cancers was OPN [44]. This glycophosphoprotein modulates multiple mechanisms of tumor-mediated immune suppression and has a pivotal role at the crossroads of inflammation and tumor progression. OPN plays an important role in tumor reparing, processes of remodelling the extracellular matrix after injury and is also responsible for protumorigenic reprogramming of TAM [45]. In experimental studies, OPN deficiency was associated with reduced immune-suppressive activity of M2 macrophages and decreased OPN/CD44 signaling lead to promotion of aggressive tumor growth as a conseqeunce of enhancement of cancer stem cell activity in the glioma perivascular niche [46]. Moreover, in patients, elevated OPN levels have been shown to correlate with poor prognosis [47].

Recently, the idea of using aptamers in neurooncology-synthetic single-strand oligonucleotydes appeared. Their high binding affinity against target protein seem to be able to inhibit malignancy features. In preclinical studies they showed effectiveness and good safety profile which should be verified in clinical studies [48].

TMZ – the monofunctional DNA alkylating agent is a drug of first choice in therapy of GBM patients. In fact, GBM response to treatment is unpredictable due to its infiltrative and complex nature. However, according to commonly used regimen recommended by Stupp, additional administration of TMZ improves the overall survival and progression-free survival in comparison to radiotherapy alone (14.6 months vs. 12.1) [49]. It is known that good clinical response to TMZ is associated with MGMT promotor methylation, however on the other hand, TMZ administration in this group of patients generates mutation phenotype and causes relapse [50]. For example, isocitrate dehydrohenase (IDH)-mutant, low grade astrocytomas treated with TMZ can reccure as more malignant tumors with DNA mismatch repair (MMR) defects and thousands of de novo mutations. Despite this, TMZ still remains the first choice alkylating drug in GBM because application of MGMT inhibitors is limited by mielotoxicity. Therefore, recurrence still remains an unresolved problem [51,52]. To sum up, paradoxically, price for inhibition of tumor growth is chang in the genomic landscape, like hypermutated phenotype, alterations in tumor subclonal architecture and promotion of cancer evolution which have an adverse impact to the therapeutic management of recurrent GBM.

In this study, TMZ exerted different effects, specific for each line, namely: up-regulation of most proteins (HROG02, HROG17), down-regulation of protein profile (T98G), loss (T98G) or appearance of some proteins (T98G, HROG02). TMZ only in lines derived from not treated patients (T98G and HROG02) but not in recurrent HROG17 line caused the appearance of TIE-2 and Thrombospondin. These proteins can shape and convert the GBM profile to a more malignant form through the following processes: aberration of communication between glioma cells and tumor microenvironment, increase expression of ATP-binding cassette (ABC) transporters that induce chemoresistance phenotype (TIE-2), intensify immunosuppression, escape fom immune supervision or creation of TGF-mediated nanotubes (Thrombospondin) [52,53]. Among „top” 10 proteins common in all lines, TMZ modulated 5 proteins: FGF,

Cathepsin s,cathepsin b, Survivin, Axl and Osteopontin in different directions. Although these proteins present a wide range of functions, all of them are engaged in immune response and create favorable conditions for the following cellular processes like: degradation of the type IV collagen and extracellular matrix, chromosomal instability, self-renewal, impaired cell-cell interactions, motility and proliferation or cell cycle (Fig. 3). These proteins are closely associated with hyperactivation of MAPK kinase pathway that probably stimulates GBM malignancy and has been recently suggested to be involved in GBM relapse [54]. Unfortunately, to this day, all attempts to achieve an inhibition of the hyperactive pathways did not give satisfactory results and has led to the zero point.

Conclusions

Although the mechanisms of glioma invasion have been investigated in various experimental studies, there is still an urgent need for an elaboration of novel panel of biomarkers that will properly characterize invasive phenotypes of GBM. All researchers hope that the succesful completion of studies will facilitate creation of personalized therapies which is so much required and improve examination of prognosis in patients with GBM.

The complexity of GBM disease and results of our analysis of cancer-related protein profile after exposition of patient-derived HROG02, HROG17 GBM lines and commercial T98G cell line to TMZ inspire to take a look at several issues that can shape a malignant character of GBM and manage its diffusely infiltration into the normal brain parenchyma leading to tumor relapse. Dynamic changes in protein profile expression observed before and after temozolomide action consisted of protein up- and down-regulation, the appearing/disappearing or stabalizing events indicate that it is impossible to point out a single protein responsible for GBM development. Since proteins create a network of interconnections and are effectors of genome instability induced by hypoxia and temozolomide [55], we suggest that (comprehensive) protein profiles have a greater credibility than some promising results of experimental *in vivo* studies dedicated to single proteins. Unfortunately, up to now their results did not improve therapeutic strategy.

A proof of this is the fact that despite remarked uniqueness of relapse HROG17 line we have detected proteins common for all lines and observed before and after TMZ action such as: FGF, Survivin, Axl, Sparc, u-plasminogen, HO-1, osteopontin, MMP-2, cathepsin s, cathepsin b. Their overlapping functions and involvement in key malignant events makes it impossible to choose one, „the most important protein” but creates a challenge to design therapy targeting all/a multitude of these proteins simultaneously.

Though relapse of GBM is an interesting subject from research and diagnostic point of view, nevertheless primary tumor, is the scaffolding for the malignant transformation. While HROG02 and commercial T98G line showed similar changes in detected proteins, the relapsed HROG17 line was the unique one. In GBM relapse line we have observed weaker cell proliferation and confluence was achieved after exposition to TMZ and separation of living cells. In this line wound healing was observed twice as fast as in HROG02

and commercial T98G line and quite different phenotype of microglial cells was found, with or without only single round active M1 polarization cells.

Finally, only in relapse HROG17 line we observed loss of activity of 7 proteins with proven role in strong dysregulation of energy metabolism and metabolism remodeling. On the other hand, detection of two key proteins in relapse GBM line such as kallikrein and thrombospondin that are involved in ECM degradation, cell-cell interaction and modulation of immunology response emphasizes a tight relationship between tumor immunology and its aberrant metabolism. In consequence, it seems that this relationship may be considered as a new direction in GBM studies.

Declarations

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Competing Interest and Funding

This work was supported by a Grant from the School of Medicine, Medical University of Silesia, Katowice, Poland (KNW-1-121/N/9/0).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Greetings

We would like to express our sincere thanks to Mrs Edyta Golińska, Mrs Anna Rynkowska, Mrs Amy Hamilton and Mr Alexander Jurkowitsch for the LeviCell equipment and support in the execution and analysis of the separation experiment.

References

1. Yang K, Wu Z, Zhang H, Zhang N, Wu W, Wang Z, et al.,. Glioma targeted therapy: insight into future of molecular approaches. Mol Cancer. 2022, 8;21(1):39.
2. Jiapaer S, Furuta T, Tanaka S, Kitabayashi T, Nakada M. Potential Strategies Overcoming the Temozolomide Resistance for Glioblastoma. Neurol Med Chir. 2018, 15;58(10):405-421.
3. Shergalis A, Bankhead A, Luesakul U, Muangsin N, Neamati N. Current Challenges and Opportunities in Treating Glioblastoma. 2018, Pharmacol Rep. 70(3):412-445.
4. Wang LB, Karpova A, Gritsenko MA, Kyle JE, Cao S. Proteogenomic and metabolomic characterization of human glioblastoma 2021, Cancer Cell. 12;39(4):509-528.

5. Pienkowski T, Kowalczyk T, Kretowski A, Ciborowski M. A review of gliomas-related proteins. Characteristics of potential biomarkers 2021, Am J Cancer Res.15;11(7):3425-3444.
6. Sareen H, Ma Y, Becker TM, Roberts TL, De Souza P, Powter B. Molecular Biomarkers in Glioblastoma: A Systematic Review and Meta-Analysis. 2022, Int J Mol Sci. 9;23(16):883.
7. Luo J, Junaid M, Hamid,N, Duan J, Yang X, Pei DS. Current understanding of gliomagenesis: from model to mechanism. 2022, Int J Med. Sci. 19(14): 2071-207.
8. Lin GS, Wang WW, Lin H, Lin RS. Bevacizumab Combined with Intensity-Modulated Radiation Therapy on Cognitive and Coagulation Function in Postoperative Glioma Patients. 2022, J Health Eng. 2022.
9. Wirsching HG, Roth P, Weller M. A vasculature-centric approach to developing treatment options for glioblastoma. 2021, Expert Opin Ther Targets. 25(2):87-100.
10. Lam KHB, Leon AJ, Hui W, Lee SC, Batruch I, Faust K, et al. Topographic mapping of the glioblastoma proteome reveals a triple-axis model of intra-tumoral heterogeneity. 2022, Nat Commun. 10;13(1):116.
11. Goenka A, Tiek D, Song X, Huang T, Hu B, Cheng SY. The Many Facets of Therapy Resistance and Tumor Recurrence in Glioblastoma. 2021. Cells. 24;10(3):484.
12. Hernández Martínez A, Madurga R, García-Romero N, Ayuso-Sacido Á. Unravelling glioblastoma heterogeneity by means of single-cell RNA sequencing. 2022, Cancer Lett, 28;527:66-79.
13. Bielecka-Wajdman AMB , Ludyga T , Smyk D, Smyk W ,Mularska M , Świderek P et al. Glucose Influences the Response of *Glioblastoma* Cells to Temozolomide and Dexamethasone. 2022,Cancer Control.29:
14. Mullins CS, Schneider B, Stockhammer F, Krohn M, Classen CF, Linnebacher M. Establishment and characterization of primary glioblastoma cell lines from fresh and frozen material: a detailed comparison PLoS One. 2013, 7;8(8):e71070.
15. Mesrati MH, Behrooz AB, Abuhamad AY Syahir A. Understanding Glioblastoma Biomarkers: Knocking a Mountain with a Hammer. Cells. 2020, 16;9(5):1236.
16. Aggarwal N, Sloane BF. Cathepsin B: multiple roles in cancer. 2014, Proteomics Clin Appl. 8(5-6):427-37.
17. Jimenez-Pascual A, Mitchell K, Siebzehnrubl FA, Lathia JD. FGF2: a novel druggable target for glioblastoma? 2020, Expert Opin Ther Targets. 24(4):311-318.
18. Zhang S, Zhang C, Song Y, Zhang J, Xu J. Prognostic role of survivin in patients with glioma. 2018, Medicine. 97(17).
19. Wei J, Marisetty A, Schrand B, Gabrusiewicz K, Hashimoto Y, Ling LY et al. Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. 2019, J Clin Invest. 2; 129(1): 137–149.
20. Kan LK, Drummond K, Hunn M, Williams D, O'Brien TJ, Monif M. Potential biomarkers and challenges in glioma diagnosis, therapy and prognosis . 2020, BMG Neurol Open 24;2(2):e000069.

21. Scholz N , Kathreena M. Kurian KM, Siebzehnrubl FA 3 Julien D. F. Licchesi JDF. Targeting the Ubiquitin System in Glioblastoma.2020, Frontiers in Oncol. 10: 574011.
22. Luo J, Junaid M, Hamid N, Duan JJ, Yang X, Sheng Pei DS. Current understanding of gliomagenesis: from model to mechanism. 2022, Int J Med Sci. 19(14): 2071–2079.
23. Doeberitz DK, Daniel Paech D, Sturm D, Pusch S, Turcan S, Saunthararajah Y. Changing paradigms in oncology: Toward noncytotoxic treatments for advanced gliomas. 2022, Int J Cancer 1; 151(9): 1431–1446.
24. Womeldorff M, Gillespie D, Jensen RL. Hypoxia-inducible factor-1 and associated upstream and downstream proteins in the pathophysiology and management of glioblastoma. 2014, Neurosurg Focus. 37(6): E8.
25. Tu J, Fang Y, Han D, Tan X, Haifeng , Xinming Wang et al. Activation of nuclear factor-κB in the angiogenesis of glioma: Insights into the associated molecular mechanisms and targeted therapies. 2021, Cell Prolif. 54(2): e12929.
26. Wesseling P, Capper D. WHO 2016 Classification of gliomas. 2018, Neuropathol Appl Neurobiol. 44(2):139-150.
27. Qiao G, Wu A, Chen X, Tian Y, Lin X. Enolase1, a moonlight protein, as potential target for cancer treatment. 2021, Int J Biol Sci, 17(14):3981-3992.
28. Su L , Yang K , Li S , Liu C , Han J , Zhang Y et al. Enolase-phosphatase 1 as a novel potential malignant glioma indicator promotes cell proliferation and migration.2018, Oncol Rep, 40(4):2233-2241.
29. Yan T, Skaftnesmo KO, Leiss L, Sleire L, Wang J, Li X et al. Neuronal markers are expressed in human gliomas and NSE knockdown sensitizes glioblastoma cells to radiotherapy and temozolomide. 2011, BMC Cancer, 11: 524.
30. Yuan J, Zhang JF, Hallahan D, Zhang Z, L He L, Gang Wu LG et al. Reprogramming glioblastoma multiforme cells into neurons by protein kinase inhibitors.2018, J Exp Clin Cancer Res.; 37: 181.
31. Ostuzzi G, Matcham F, Dauchy S, Barbui C, Hotopf M. Antidepressants for the treatment of depression in people with cancer. 2018, Syst Rev. 23;4(4):CD011006.
32. Bielecka AM, Obuchowicz E.Antidepressant drugs as a complementary therapeutic strategy in cancer . 2013, Exp Biol Med. 1;238(8):849-58.
33. Kim Y, Varn FS, Park SH, Yoon B, Park HR, Lee CH et al. Perspective of mesenchymal transformation in glioblastoma. 2021, Acta Neuropathol Commun. 9:50.
34. Xiong W, Li C, Kong G, Wan B, Wang S, Fan J. Glioblastoma: two immune subtypes under the surface of the cold tumor. 2022, Aging. 23;14(10):4357-4375.
35. Quesnel A, Karagiannis GS, Filippou PS. Extracellular proteolysis in glioblastoma progression and therapeutics. 2020, Biochim Biophys Acta Rev Cancer. 1874(2):188428.
36. Drucker KL, Paulsen AR, Giannini C, Paul A. Decker PA, Blaber SI et al. Clinical significance and novel mechanism of action of kallikrein 6 in glioblastoma. 2013, Neuro Oncol, 15(3): 305–318.

37. Joseph JV, Magaut CR, Storevik S, Geraldo LH, Mathivet T, Latif MA, et al.. TGF-β promotes microtube formation in glioblastoma through thrombospondin 1. 2022, Neuro Oncol. ;24(4):541-553.
38. Zhang Q, Wang J, Yao X, Wu S, Tian W, Gan C et al.. Programmed Cell Death 10 Mediated CXCL2-CXCR2 Signaling in Regulating Tumor-Associated Microglia/Macrophages Recruitment in Glioblastoma. 2021, Front Immunol. 24;12:637053
39. Maas SLN, Abels ER, Van De Haar LL, Zhang X, Morsett L, Sil S et al. Glioblastoma hijacks microglial gene expression to support tumor growth. 2020, JNeuroinflammation. 16;17(1):120.
40. Xu C，Xiao M, Li X，Lei Xin L, Song J，3 Zhan, Z et al. Origin, activation, and targeted therapy of glioma-associated macrophages 2022, Front Immunol. 13: 974996.
41. Wohlleben G, Scherzad A, Güttler A, Vordermark D, Kuger S, Flentje M et Osteopontin mediates glioblastoma-associated macrophage infiltration and is a potential therapeutic target. 2019, J Clin Invest. 129(1): 137–149.
42. Moorman HR, Poschel D John D. Klement JD, Lu Ch, Redd PS, Liu K. Osteopontin: A Key Regulator of Tumor Progression and Immunomodulation. 2020, Cancers. 12:3379.
43. Genard G, Lucas S, Michiels C . Reprogramming of Tumor-Associated Macrophages with Anticancer Therapies: Radiotherapy versus Chemo- and Immunotherapies.2017, Front Immunol. 8:28.
44. Alexander Pietras A, Katz M, Ekström EJ, Wee B, Halliday JJ, Kenneth L. et al. Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. 2014, Cell Stem Cell. 6; 14(3): 357–369.
45. Polat B, Wohlleben G , Kosmala R ，Lisowski D ，Mantel F ，Lewitzki V et al.. Differences in stem cell marker and osteopontin expression in primary and recurrent glioblastoma. 2022, Cancer Cell Int. 19;22(1):87.
46. Amero P, Khatua S, Rodriguez-Aguayo C, Lopez-Berestein G. Aptamers: Novel Therapeutics and Potential Role in Neuro-Oncology. 2020, Cancers (Basel). 9;12(10):2889.
47. Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B, et al., Effect of Tumor-Treating Fields Plus Maintenance Temozolomide vs Maintenance Temozolomide Alone on Survival in Patients With Glioblastoma: A Randomized Clinical Trial. 2017, JAMA . 19;318(23):2306-2316.
48. Butler M, Pongor L, Su YT, Xi L, Raffeld M, Quezado M et al. MGMT Status as a Clinical Biomarker in Glioblastoma. 2020, Trends Cancer. 6(5):380-391.
49. Touat M, Li YY, Boynton AN, Spurr LF, Iorgulescu JB, Bohrson CL et al. Mechanisms and therapeutic implications of hypermutation in gliomas. 2020, Nature. 580(7804):517-523.
50. Justin V Joseph, Capucine R Magaut, Simon Storevik, Luiz H Geraldo, Thomas Mathivet, Md Abdul Latif et al. TGF-β promotes microtube formation in glioblastoma through thrombospondin 1.2022, Neurooncology. 24;4, 541-553.
51. Sho T , Toshiya I, Taishi Ti, Shingo T, Farida G, Hemragul S, et al. Tumor Microenvironment in Glioma Invasion. 2022, Brain Sci. 12(4): 505.

52. Codrici E, Popescu ID, Tanase C, Enciu AM. Friends with Benefits: Chemokines, Glioblastoma-Associated Microglia/Macrophages, and Tumor Microenvironment. 2022, Int J Mol Sci. 23(5): 2509.
53. Aasland D, Goetzinger L, Hauck L, Berte N, Meyer J,Effenberger M et al. Temozolomide Induces Senescence and Repression of DNA Repair Pathways in Glioblastoma Cells via Activation of ATR–CHK1, p21, and NF-kB. 2019, Cancer Res:71(1): 99-113.

Figures

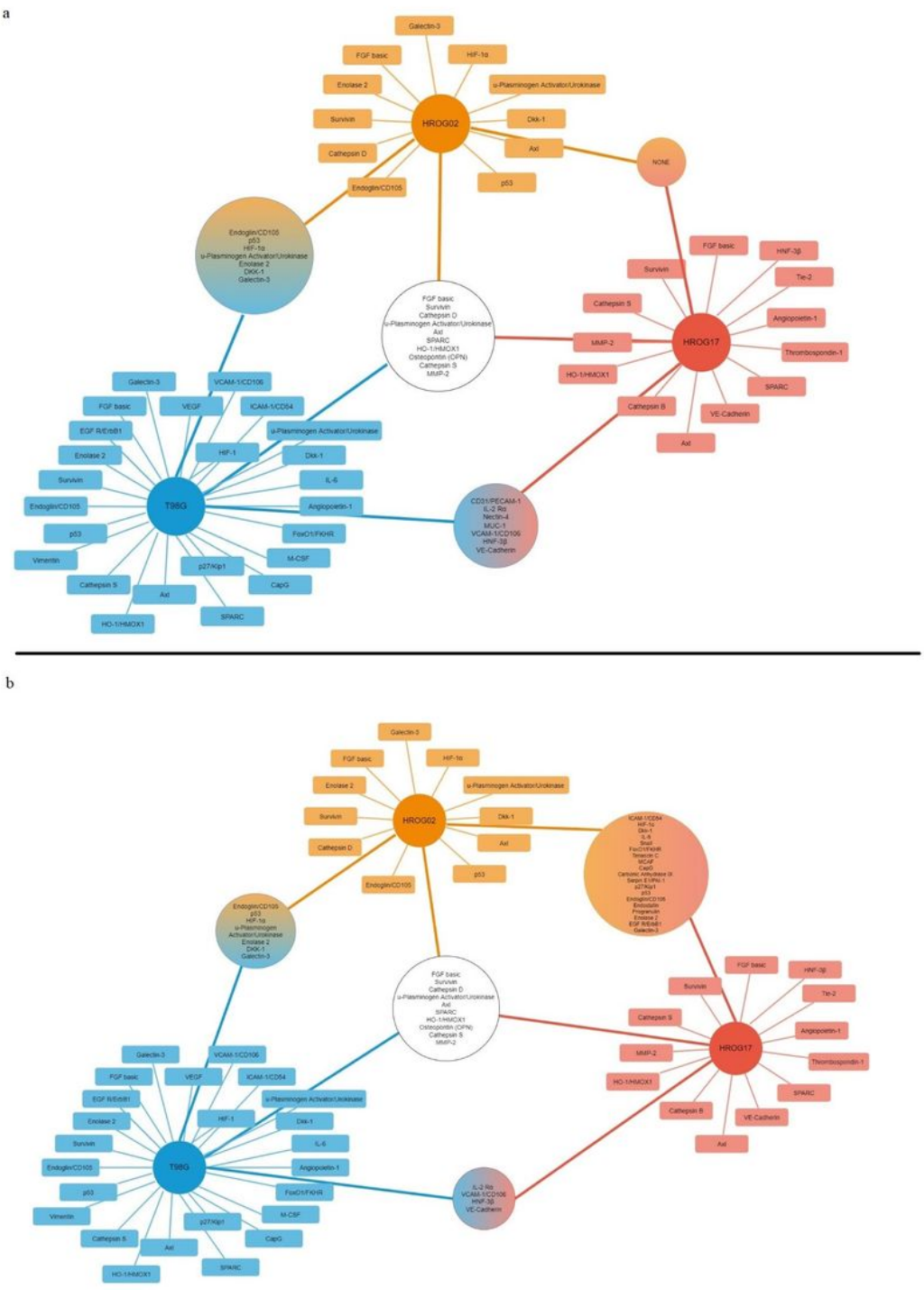

Figure 1

Cancer- related proteins common and unique for three analyzed lines: in control (A) and after Temozolomide action.

A

PROTEIN NAME	FOLD CHANGE	PROTEIN NAME	FOLD CHANGE
Reference Spots	11%	IL-6	-84%
Galectin-3	14%	Dkk-1	-76%
FGF basic	-35%	PDGF-AA	-76%
EGF R/ErbB1	-44%	u-Plasminogen Activator/Urokinase	-78%
Enolase 2	-72%	Tie-2	142%
Progranulin	-18%	ErbB4	-43%
Survivin	-47%	FoxC2	-31%
Endostatin	-20%	Progesterone R/NR3C3	-82%
Cathepsin D	58%	α-Fetoprotein	-42%
Endoglin/CD105	-44%	E-Cadherin	-12%
p53	-65%	HIF-1α	-92%
Vimentin	217%	CXCL8/IL-8	-88%
Cathepsin S	-68%	Angiopoietin-like 4	-49%
MMP-2	2%	CEACAM-5	-56%
HO-1/HMOX1	-58%	ErbB3/Her3	-36%
Cathepsin B	-24%	VEGF	-92%
Axl	-25%	ENPP-2/Autotaxin	-61%
p27/Kip1	-61%	ICAM-1/CD54	-80%
Serpin E1/PAI-1	-52%	eNOS	-39%
Osteopontin (OPN)	-46%	Amphiregulin	-37%
Carbonic Anhydrase IX	16%	HNF-3β	-60%
VE-Cadherin	-15%	Prostasin/Prss8	-72%
SPARC	-74%	EpCAM/TROP1	-73%
CapG	-83%	Mesothelin	-71%
Thrombospondin-1	261%	ERα/NR3A1	-72%
CG α/β (HCG)	-39%	CA125/MUC16	-68%
M-CSF	-84%	VCAM-1/CD106	-91%
Tenascin C	-19%	Prolactin	-69%
FoxO1/FKHR	-76%	MSP/MST1	-80%
Decorin	-38%	BCL-x	-79%
GM-CSF	3%	Kallikrein 3/PSA	-77%
Snail	-65%	MUC-1	-75%
HGF R/c-Met	-31%	Nectin-4	-82%
Angiopoietin-1	-73%	IL-2 Rα	-77%
DLL1	-58%	CD31/PECAM-1	-66%
ErbB2	-70%	Reference Spots	13%
MMP-3	-80%		

B

PROTEIN NAME	FOLD CHANGE
Reference Spots	15%
Galectin-3	49%
FGF basic	393%
EGF R/ErbB1	-17%
Enolase 2	-35%
Progranulin	-7%
Survivin	46%
Endostatin	296%
Cathepsin D	74%
Endoglin/CD105	121%
p53	66%
Vimentin	59%
Cathepsin S	2%
MMP-2	-4%
HO-1/HMOX1	0%
Cathepsin B	60%
Axl	233%
p27/Kip1	146%
Serpin E1/PAI-1	16%
Osteopontin (OPN)	12%
Carbonic Anhydrase IX	30%
SPARC	-10%
CapG	-1%
Thrombospondin-1	294%
CG α/β (HCG)	94%
M-CSF	-47%
Tenascin C	37%
FoxO1/FKHR	-68%
Snail	85%
HGF R/c-Met	-4%
ErbB2	210%
MMP-3	-4%
IL-6	454%
Dkk-1	401%
PDGF-AA	119%
u-Plasminogen Activator/Urokinase	-36%
Tie-2	246%
HIF-1α	-58%
CXCL8/IL-8	-35%
Angiopoietin-like 4	-67%
VEGF	-46%
ICAM-1/CD54	715%
Prolactin	0%
Reference Spots	-9%
Reference Spots	25%

C

PROTEIN	FOLD CHANGE
Reference Spots	102%
FGF basic	223%
Survivin	135%
Cathepsin S	114%
MMP-2	229%
HO-1/HMOX1	150%
Cathepsin B	178%
Axl	119%
Osteopontin (OPN)	84%
VE-Cadherin	57%
SPARC	140%
Thrombospondin-1	89%
Angiopoietin-1	115%
u-Plasminogen Activator/Urokinase	97%
Tie-2	222%
HNF-3β	117%
VCAM-1/CD106	135%
MUC-1	113%
Kallikrein 5	128%
Nectin-4	60%
IL-2 Rα	186%
CD31/PECAM-1	76%

Figure 2

Expression profile of cancer- related proteins after exposition to temozolomide (TMZ) in A. T98G, B. HROG02 C. and HROG17 cell lines. The numbers (in percent) show the change fold in respect to the control line that was not treated with TMZ.

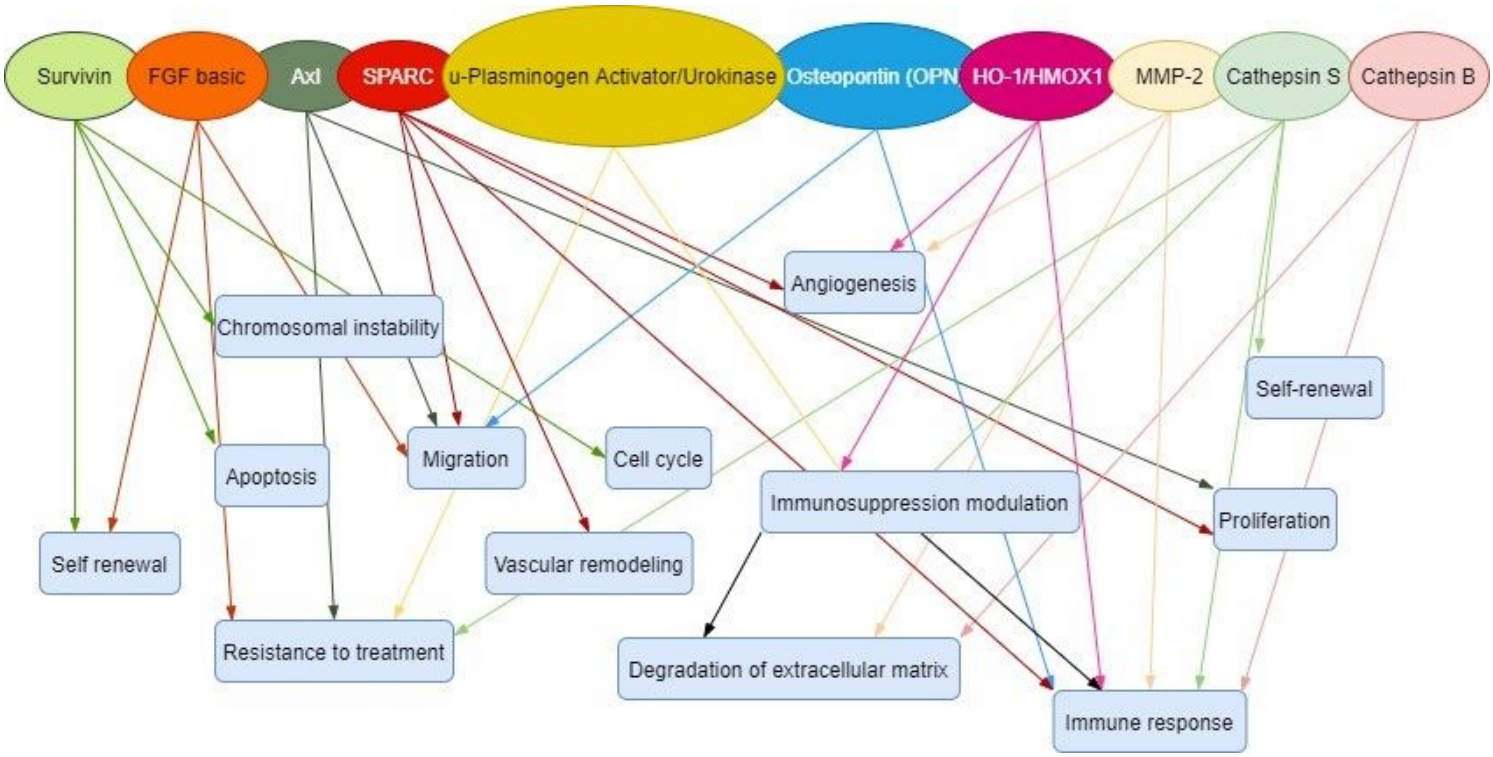

Figure 3

Common proteins and their functions in cancer cell.

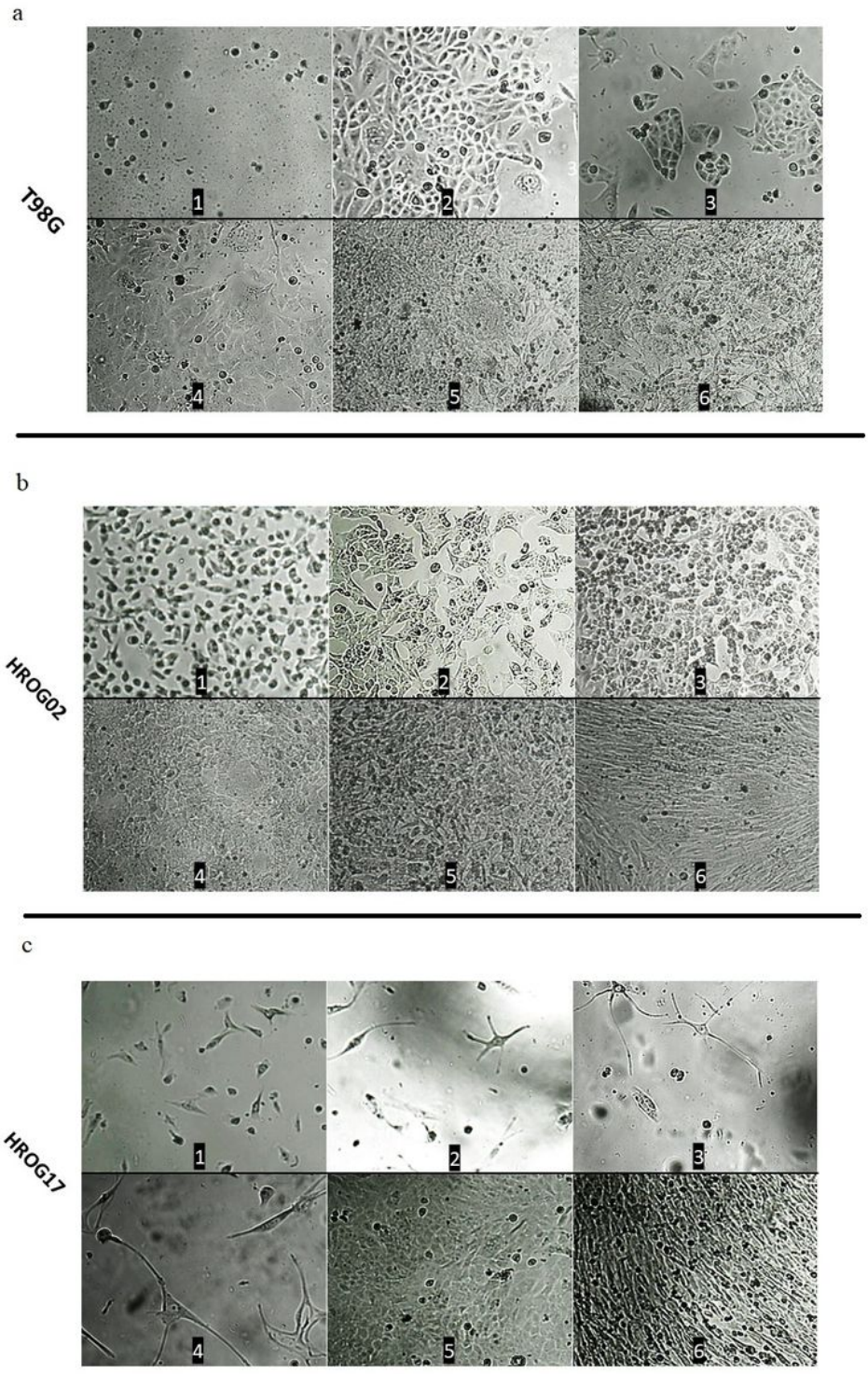

Figure 4

Separation test.

Viable cells separated from dead cells after exposition to cytotoxic TMZ concentrations showed differences in the rate of dynamic confluence. Viable cells from two lines: primary HROG02 (B) and commercial T98G (A) line reached confluence faster (after 5 vs. 8 days, respectively). In contrast

remaining viable cells from HROG17 (C) did not reach a confluence before day 12. Moreover, HROG 17 cells were characterized by completely different phenotype than other lines. These cells were spindle-shaped with long processes and round, activated cells were not noticed.

Each line reached full confluence at its own pace despite prior TMZ exposure. After 3 weeks, cells of all lines did not detach from the plate bottom as it should be when culture is conducted for long time but they formed multi-layer compact structures. (Fig. 4A,B,C).

Number of picture:

1-cell culture in 3 day; 4- cel culture in 10 day

2- cel culture in 5 day; 5- cel culture in 12 day

3- cel culture in 8 day; 6-cell culture after 3 weeks

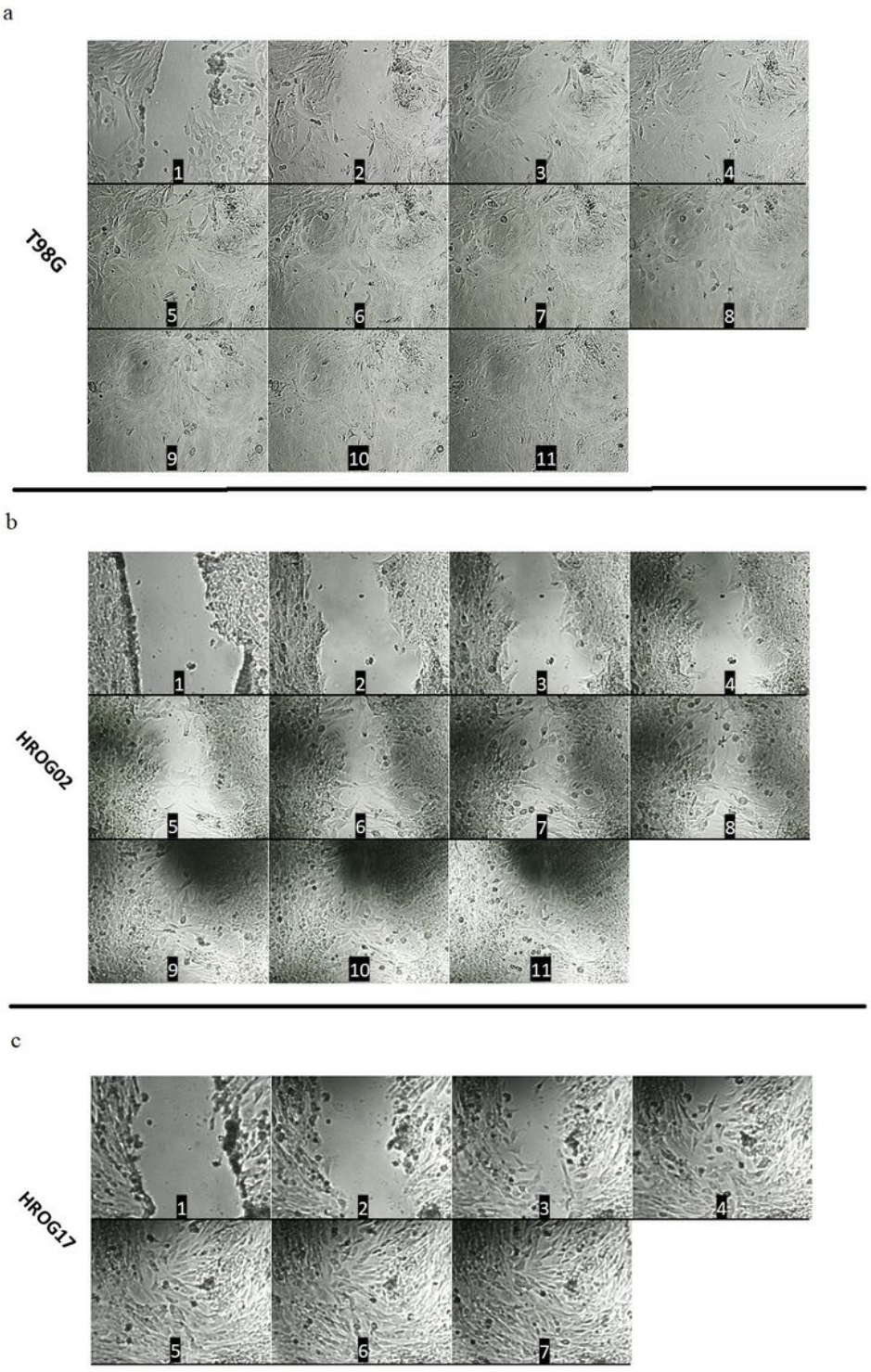

Figure 5

Migration of cells in wound healing test.

A sequence of representative images at an interval of 100 minutes in our wound healing assay was carried out in three lines: T98G (A), HROG02 (B) and HROG17 (C) using July stage cell analyzer. Migration of T98G and HROG02 cells was similar. Time needed for wound healing, considered as an index of cell

migration, was about 30 hours for T98G and HROG02 cells and 15 hours for HROG17 cells. In cultures of HROG02 and T98G cell lines, shortly before the wound was completely closed, round cells with the phenotype of activated microglia appeared. Such cells were not present in HROG17 cultures.

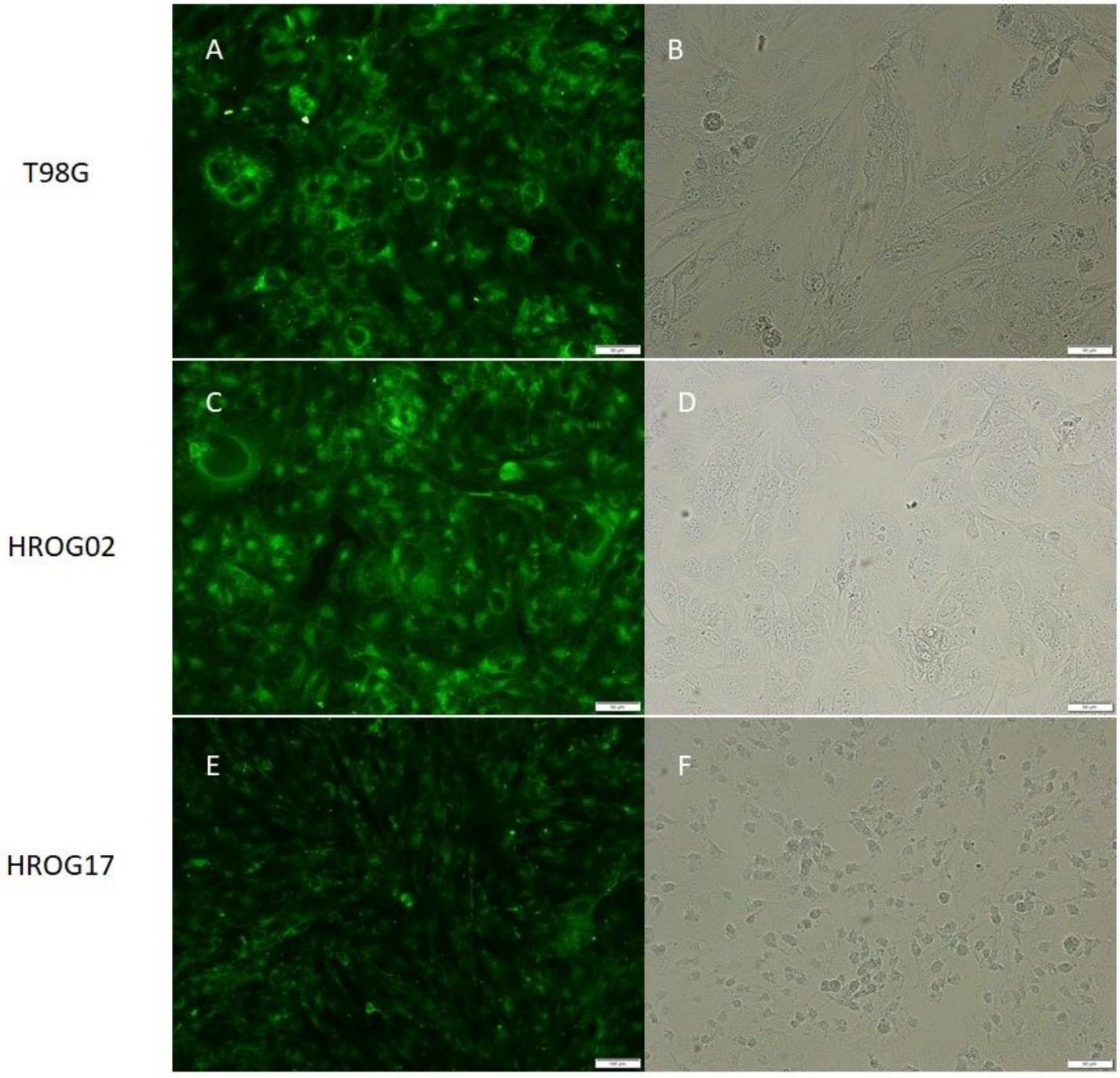

Figure 6

Microglia phenotype

Microscopic observations using agglutinin *Ricinus communis* showed significant differences in morphology of GBM microglia cells especially between relapse HROG17 and HROG02/T98G lines. In the relapse line, as opposed to the other two, cells in the resting phase with numerous processes were visible.

In commercial and primary HROG02 line, soma cells were round and presented M1 activated phenotype (area=3385 and 3776 μm^2 in T98G and HROG02 line, respectively; and 133 μm^2 in HROG17) (OlympusBX43 Microscope and 20x magnification lens).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstract2.jpg