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Novel Approaches for Glioblastoma Treatment: Focus on Tumor Heterogeneity, Treatment Resistance, and Computational Tools

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Abstract

Background: Glioblastoma (GBM) is a highly aggressive primary brain tumor. Currently, the suggested line of action is the surgical resection followed by radiotherapy and treatment with the adjuvant temozolomide (TMZ), a DNA alkylating agent. However, the ability of tumor cells to deeply infiltrate the surrounding tissue makes complete resection quite impossible, and in consequence, the probability of tumor recurrence is high, and the prognosis is not positive. GBM is highly heterogeneous and adapts to treatment in most individuals. Nevertheless, these mechanisms of adaption are unknown.

Recent Findings: In this review, we will discuss the recent discoveries in molecular and cellular heterogeneity, mechanisms of therapeutic resistance, and new technological approaches to identify new treatments for GBM. The combination of biology and computer resources allow the use of algorithms to apply artificial intelligence (AI), and machine learning (ML) approaches to identify potential therapeutic pathways and to identify new drug candidates.

Conclusion: These new approaches will generate a better understanding of GBM pathogenesis and will result in novel treatments to reduce or block the devastating consequences of brain cancers.

Keywords

Cancer stem cells; tunneling nanotubes (TNTs); biomarkers; gap junctions; artificial intelligence

Introduction

Malignant tumors affecting the central nervous system (CNS) are one of the most feared types of cancer. Less than 2% of all cancer aggressively affect the brain (1, 2). According to the World Health Organization (WHO), gliomas are classified in astrocytomas,

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oligodendrogliomas, oligo-astrocytomas, and GBM base on the histopathological and the clinical features (3). GBM arises within the brain parenchyma, especially in the dura mater and calvarium, which is thought to seed the extracranial space with tumor cells (4). GBM is well known as one of the most aggressive, frequent, and devastating types of glioma, corresponding to 52% of all primary brain tumor cases (5). GBM incidence increases of 0.7% every 11 years (6) and it is greater in men (roughly 7.7 per 100,000) than in women (5.61 per 100,000), making this type of cancer a serious health problem (1).

The most typical characteristics of GBM are robust angiogenesis, intense resistance to apoptosis, necrogenesis, genomic instability, heterogeneity, and adaptation to treatment (7–9). Formerly, GBM tumors were classified based on the morphological appearance and normal glial development in the brain (10). When these tumors become more aggressive, the morphology of the cells changes and they are less differentiated under a microscope (11). Currently, GBM classification is based on both phenotype and genotype expression (12). GBM is recognized as a diffuse astrocytoma and it could be considered as isocitrate dehydrogenase (*Idh*)-wildtype type of tumor, also known as a primary GBM (90% of the cases), or *Idh*-mutant tumor identified as secondary GBM (10% of the cases) (3). Besides the numerous advances in biomedical research, surgical techniques, diagnosis, and treatment, both types of GBM have a poor prognosis and 14.6 months of survival rate. GBM has a dramatical annual incidence of 3.19 per 100,000 (7). Currently, surgical resection combined with radiotherapy plus concomitant treatment with the adjuvant temozolomide (TMZ) is the standard care in patients younger than 70 years old with newly diagnosed GBM (13–16). Furthermore, extracranial metastasis in GBM patients is extremely rare (<2% of the cases), due to the presence of unique barriers such as the blood-brain barrier (BBB), the dura mater and the thickened basement membrane of the blood vessels, which prevent the hematogenous and lymphatic spread of intracranial tumor cells and suppression of extracranial GBM cells growth by the immune system (17). Also, lack of extracellular matrix proteins prevents tumor invasion in the surrounding connective tissue, and consequently hematogenous and lymphatic spread (2). However, genetic and molecular factors that predict extracranial invasion remain unclear and require further investigation.

Only recently, tumor heterogeneity becomes a hallmark of GBM and it is influenced by genetic, epigenetic and metabolic biomarkers in cancer cells, with different degrees of differentiation and tumor microenvironment. The unique cellular composition of these kinds of tumor gives them the capacity to become highly infiltrative and invasive, to have nuclear atypia, to increase proliferation, to generate microvascular hyperplasia, and necrotic foci (10, 18). Specifically, glioblastoma stem cells (GBSCs) have an important role for survival and adaptation. GBSCs are one of the major contributors to the molecular and cellular heterogeneity observed in GBM. GBSCs are capable of self-renewal and are responsible for therapeutic resistance and tumor recurrence (19–22). The hierarchical GBSC cancer model proposes that tumor arises from GBSCs generated by mutations in either normal embryonic stem cells or progenitors cells or by dedifferentiation, resulting in cells with uncontrolled growth and propagation (23). After surgical resection of the tumor and radio/chemotherapy therapy, the remaining GBSCs from the border of the tumor can repopulate the tumor suggesting the presence of aggressive GBSCs at the border or infiltrated into healthy tissue (22, 24, 25). A recent study performed in 10 samples of tumor and their primary derived

GBSCs, identified that both GBM and matched GSCs have a recurrent copy number of genetic alterations, in chromosome 7 polysomy, chromosome 10 monosomy, and chromosome 9p21 deletions, which are typical features of primary GBM, essential for gliomagenesis (26). Thus suggests a condition of strong genomic heterogeneity in GBM as in GBSCs (26, 27).

Genetics also play a key role in the GBM heterogeneity. Mutations in genes encoding proteins can increase the resistance to standard radio and chemotherapy. One of the genetic alterations that have an important impact in GBM resistance to treatment involves the mutation of the epidermal growth factor receptor *(Egfr)* gene, that occur in 36%–60% of primary GBM resulting in different modulation of EGFR signaling and related receptors (22, 28, 29). Also, the homozygous deletion of the cyclin-dependent kinase inhibitor 2 A *(Cdkn2a)* gene, which encodes the *p16INK4a* and *p14ARF* tumor suppressors, is more observed in primary than in secondary GBM resulting in aberrant cell proliferation (30–32). Other important genetic alterations include *loss of heterozygosity (LOH) of chromosome 10*, which is present in up to 70% of primary GBM (31, 33), and the isocitrate dehydrogenase *(Idh)* gene mutations resulting in increased DNA hypermethylation (22, 34). Furthermore, for primary GBM a miRNAs study showed alterations in both genes, *Tp53* and O^6-methylguanine DNA methyltransferase *(Mgmt)*, which are major features in GBM pathogenesis, apoptosis, and treatment resistance that affect the survival of patients with primary GBM (35). From the epigenetic point of view, the resistance to treatment has been studied trough the expression of the DNA repair protein AlkB homolog 2 (Alkbh2) in GBM cell lines after TMZ exposure by real time polymerase chain reaction (qRT-PCR). Alkbh2 is regulated by the *p53* pathway and it has an increased resistance to methylating agents like TMZ (36). Furthermore, it was found that GBM cells overexpress *Alkbh2* after TMZ exposure, enhancing the resistance to methylating agents, including TMZ (36). The susceptibility to cancer and treatment resistance has also been studied for the expression of the protein glutathione-S-transferase-π (GSTP1) mutation in several kinds of tumors (37–40). In GBM, GSTP1 is highly heterogeneously expressed and plays an important role in the protection of cells against damage from free radicals and also influences cytotoxicity to chemotherapeutics agents as TMZ (41, 42). A study of 61 astrocytic tumor samples from WHO grade II-IV showed that there were no differences in GSTP1 mRNA expression between diffuse astrocytomas, anaplastic astrocytomas, or GBM. No difference was seen between secondary GBM before and after radio-/chemotherapy, suggesting that glioma chemoresistance is probably multifactorial and GSTP1-independent (38).

As indicated above, the cellular heterogeneity, infiltrative and accumulative capability of GBM cells make complete surgical resection of the tumor almost impossible. Advances in genomic sequencing and transcriptomic profiling reveal heterogeneity of GBM, dividing it into molecular subtypes: proneural, neural, classical and mesenchymal (14, 43). The cellular composition of GBM subtypes shows marked heterogeneity; the proneural class is highly enriched with oligodendrocytes but not with astrocytes, whereas the classical GBM is strongly associated with astrocytes (43). The neural subtype is associated with oligodendrocytes and astrocytes differentiation but also is enriched with neuron products (43). The mesenchymal subtype is strongly associated with astroglial cells and tumor associated macrophages (TAMs) (43–45). Each subtype of GBM is also characterized by

different transcriptional profile. The heterogeneity in GBM was analyzed according to the gene expression of platelet-derived growth factor receptor alpha *(Pdgfra)*, isocitrate dehydrogenase *(Idh1)*, *Egfr* and neurofibromatosis type I *(Nf1)* (43). It was found that genes in normal cell types show a strong relationship between the GBM subtype and different neural lineages, where classical subtype showed a good response to therapy. Instead, the proneural subtype had the worst prognosis — these analyses associated the proneural subtype of GBM with high levels of *Pdgfra* and *Idh1 m*utations (43, 46, 47). In the mesenchymal subtype of GBM, *Nf1* expression was predominantly (43, 45). Furthermore, in three *Idh* wild-type *GBM-intrinsic* gene expression subtypes, *Nf1* deficiency results in increased macrophage/microglia infiltration (48). In patients with mesenchymal GBM, it was identified that the transcriptional coactivator with PDZ-binding motif (TAZ), is highly associated with the mesenchymal network, silencing of TAZ in mesenchymal GBSCs decreased expression of mesenchymal markers, invasion, self-renewal, and tumor formation (49, 50). The *Egfr* is also heterogeneously expressed in proneural, classical and mesenchymal subtypes (51). But the high levels of *Egfr* amplification was observed in 97% in the classical subtype of GBM (43). In the case of the neural subtype of GBM, it was found the expression of neuron markers such as Neurofilament (NEFL), Gamma-Aminobutyric Acid Type A Receptor Alpha1 Subunit (GABRA1), Synaptotagmin 1 (SYT1), and Solute carrier family 12 member 5 (SCL12A50) (43). A detail genomic and genetic classification of the different subtypes of GBM helps to understand the molecular heterogeneity and the pathways involved to design new therapies correlated with glioma's type.

The high heterogeneity of GBM explains the poor response to treatment (radio- and chemotherapy), because of the presence of cellular subpopulations (44). On the other hand, cells from the same tumor may express different mutations or shown distinct phenotypic or epigenetic stages. Single-cell RNA sequence of five types of GBM cells shows that the GBM subtype is variable express across individual cells within the tumor and demonstrates that increased heterogeneity was associated with decreased survival, affecting the potential prognosis implications of the intratumoral heterogeneity (52). The quantification of tumor growth kinetics shows two GBM patient subpopulations viewing one faster growing yet more responsive with increased survival and one slower growing yet less responsive survival with shorter survival, suggesting that many patients who receive standard-of-care treatments may get better benefit from select alternative treatments (7).

Furthermore, the high levels of the non-neoplastic cell population microenvironment include TAMs. They are strongly correlated with intratumoral vascular density (53). Specifically, TAMs differentiate into M2 macrophages, act as protumoral macrophages, and contribute to disease progression. It was found by immunofluorescence staining an association of *Nf1* deficiency with infiltration of TAMs/microglia, suggesting that *Nf1* deactivation may promote macrophages/microglia recruitment in tumors (48). The DNA repair pathways and intratumoral heterogeneity in GBM tumors show that transcriptional heterogeneity was identified in 40% of the cases with variability in *Mgmt* methylation status in 14% of the cases (54). A good example of intertumoral heterogeneity is the communication of glioma cells with cells resistant to radio or chemotherapy through long membrane extensions called

tumor microtubes or tunneling nanotubes (TNTs) (55–58). TNTs contributed after one cycle of TMZ chemotherapy to the tumor cells chemotherapy resistance (56).

One of the major contributors to chemotherapy adaptation or resistance is the enzyme MGMT. MGMT prevents apoptosis mediated by damaged DNA repairing damaged DNA (59). Besides the poor knowledge of the mechanisms of tumor survival and adaptation to treatments, we recently discovered an additional mechanism of MGMT mediated resistance mediated by TNTs. TNTs are long-range communication systems between cells allowing the transfer of protective factors such as MGMT. TNTs enable resistant cells radiotherapy and TMZ treatment and to share MGMT in order to protect tumor cells that are negative for MGMT. These novel mechanisms and how adaptation occur will be discussed below.

Only recently, several communication systems within a tumor system have been discovered including, localized gap junction (GJ), hemichannels and TNTs; TNTs are the focus of this special issue. These include interactions between tumor subpopulations, interactions between the tumor and its stroma surrounding, interactions between the tumor and the immune components, and even interactions between the tumor and the local parenchymal compartment, most mediated by TNTs and GJ (55). Thus, current efforts are being directed towards personalized treatment through blocking prime signaling pathways in gliomagenesis and understanding acquired resistance. Advances in cell-to-cell communication are contributing in the discovery of new therapies and drugs using computational approaches such as artificial intelligence (AI) and machine learning (ML). AI and ML can analyze different database to identify new affected pathways and to design new potential drug (60).

Glioblastoma Heterogeneity

GBM is a heterogeneous tumor at the histological, cellular, and molecular level (see Fig. 1) (9, 10, 18). Understanding tumor heterogeneity will help to design efficacious therapies for the treatment and avoid tumor regrowth. GBM tumors have distinct phenotypes that are critical for adaptation to fluctuations in their environment which are reflected in their morphology, growth rates, tumor progression, and treatment response (radio- or chemo-therapy). Over the past decade, GBM tumors have increasingly recognized for the complexity in the cell population (see Fig. 1). From this perspective, the biology of a tumor cannot be studied, such as an individual cell type or a particular microenvironment (44, 61). To explain the intra-tumoral heterogeneity, two mechanisms have been proposed. One is the clonal evolution model that proposes cumulative genetic or epigenetic mutations in individual normal cells, leading the formation of cancer cells that clonally expand into cells with tumorigenic potential (23, 62, 63). The second model proposed is the cancer stem cell model that suggests that only a subset of cancer cells possess indefinite self-renewal to initiate and maintain tumor growth (63). Therefore, the tumorigenic GBSCs differentiate into nontumorigenic GBSCs, creating a hierarchical organization. The differentiation of GBSCs provides a mechanism for generating phenotypic and functional heterogeneity that can be attributed to clonal evolution environment differences (23). This model suggests that in some cancers, only a minority of cells can proliferate extensively and some therapies that shrink tumor might not be curative because they fail to eliminate GBSCs (62).

As shown in Fig 1, GBM intratumoral heterogeneity provides cellular niches enriched with distinct cells with different phenotypic properties, transient quiescence, self-renewal, adaptation to hypoxia, and resistance to chemotherapy and radiation (9, 64). The tumor microenvironment in GBM is constituted by highly proliferative malignant astrocytoma cells, immune cells (lymphocytes, tumor-associated macrophages or TAMs and microglia), neurons, endothelial cells, and GBSC (65). The immune cells, principally macrophages, are one of the most relevant in the tumor because they constitute up to 30–40% of the mass of the tumor (66), showing an increase in their population with the severity of the glioma (67). TAMs are highly plastic cells showing a reciprocal interaction with neoplastic tumor cells to promote growth and progression (44); they are strongly correlated with intratumoral vascular density. Besides TAMs, T cells in GBM represent less than 0.25% of total tumor cells isolated from hGBM biopsy samples as examined by flow cytometry (68). Due to this intrinsic tumor heterogeneity, immune cells can be used as a potential target for new GBM drug development. Also, a novel layer of complexity is the cell-to-cell communication among these cells that allows signaling molecules to spread carcinogenesis and tumor resistance, including GJ, HC, and TNTs.

GBSCs are determining factors that influence intratumoral heterogeneity, and their differentiation contributes to the response to therapy, drug resistance, and prognosis. According to the GBSC hypothesis, tumor stem cells lose self-renewal, and tumorigenic potential, generating a diverse progeny of the tumor bulk (69), which initiates tumor formation (70). These cells can originate phenotypically diverse cancer cells that are situated in specialized locations where the interaction with the microenvironment regulates their behavior contributes to the molecular and cellular heterogeneity in GBM tumors. GBSCs can be classified as cancer stem-like cells (self-renew and give rise to differentiated progeny), cancer-initiating cells (initiate a tumor), and cancer-propagating cells (propagate tumor) (71). These cells cannot be studied in detail because most of the GBM tissues contain multiple populations of cells that express different markers. Nonetheless is possible to validate them with GBSCs enriched methods that allow separating the tumorigenic and non-tumorigenic populations using specific GBSCs biomarkers as Sox2, Nanog, Olig2, Myc, Musashi1, Bmi1, Nestin and inhibitors of differentiation protein 1 (Id1), Cd133, Stat3 (23). GBSCs have the potential to differentiate into astrocytes, oligodendrocytes, and neurons (72).

Tumorigenic GBSCs contribute to tumor initiation, infiltration, therapeutic resistance, and tumor recurrence after surgery (73). Importantly, GBSCs correlated with the other stem cells, and cancer stem cells have not got survival ability and specific homing (74). GBSCs could be found in both hypoxic and vascular microenvironments within tumors (perivascular niche), creating a connection between the normal neural stem/progenitors and the vasculature (75). In particular, the core region of the tumor shows high proliferation capacity and clonogenic ability, and the low expression of the differentiation markers and the genetic abnormalities are not shared with the tumor periphery. The core region also shows highly hypoxic conditions, with high enrichment of GBSCs and expression of immature markers such as CD133 and Nestin (75, 76). The intermediate layer of the tumor is hypoxic *too* and enriched with GBSCs; it shows the expression of mixed lineage markers. The periphery of the tumor is marked by the high vascularization, the rare occurrence of GBSCs, the

expression of differentiation markers, the low-level proliferation index, and the clonogenic ability (74). This intratumoral GBSCs heterogeneity ensures metabolic adaptations to support tumor growth in diverse tumor microenvironments (77). The complex subpopulation dynamics within the heterogenous intratumoral environment was characterized by microRNA expression and secretion in a phenotypically diverse subpopulation of GBSCs (78). The data indicated that phenotype-linked transcriptomics of GBSCs overlapped with anatomic tumor site, with mesenchymal-like/nodular signatures in perinecrotic zones and with a proneural-like/invasive signature in infiltrating areas of the tumor. GBSCs shape and adapt to microenvironmental conditions, and the complex intratumoral architecture arises from the co-existence of diverse GBSCs within individual tumors (78).

GBM intratumoral heterogeneity and treatment adaptation are one of the major barriers for the development of new effective therapies. This is partially due to the tumor-initiating cells (TICs), a subset that contains highly tumorigenic GBSCs. TICs are highly resistant to conventional therapies and therefore, thought to contribute to recurrent GBM (79). Furthermore, GBSC clones from patient samples with extensive molecular and phenotypic variability among clones have a range of responses to radiation and drugs. This widespread variability was observed as a continuum of multitherapy resistance phenotypes linked to a proneural-mesenchymal shift in the transcriptome (80). Multitherapy resistance was associated with a semi-stable cell state that was characterized by an altered DNA methylation pattern at promoter regions of mesenchymal master regulators and enhancers. The gradient of cell states within the glioma-initiating cell (GIC) compartment constitutes a distinct form of heterogeneity (80). A better understanding of the intratumoral heterogeneity in GBM is critical to establish faithful models and develops new therapies to treat this complex disease. We propose that GBM heterogeneity is due to TNTs communication and the presence of soluble vesicles.

MGMT Tumor Distribution in Glioblastoma

The current treatments for GBM most of time encounter resistance or adaptation to different chemotherapeutic agents, including alkylating agents, which are highly reactive molecules that promote cell death by binding to DNA. O-6-methylguanine is one of the products formed in the DNA reaction of alkylating agents and plays a key role in the initiation of mutations and the cellular cytotoxic effect of these agents (81). One of the most used alkylating agents, for the treatment of GBM, is TMZ. Therapy of malignant GBM relies on treatment with O-6-methylating agent TMZ simultaneous with ionizing radiation. TMZ is a small lipophilic molecule which can cross the BBB (82) and methylate DNA at the N-7 (70%), N-3 (9%) and O-^{6} (6%) positions of guanine residues trigging cell death (83). MGMT normally reverses the effect of chemotherapy by restoring the guanine from O-6-methylguanine. Following the repair reaction, MGMT becomes inactivated, ubiquitinated, and finally proteasomal degraded. The amount of MGMT per cell is an important determinant for the ability of cancer cells to evade alkylating agent-induced cell death, and strongly impacts the success of anticancer therapy (7). Human DNA methylation describes the covalent addition of a methyl group preferentially at 5’-position of a cytosine or guanine nucleotide (84). When *Mgmt* promoter is silenced through methylation, the MGMT enzyme is reduced (<30 fmol/mg protein), and DNA cannot be repaired, increasing the sensitivity to

the alkylating agent and enhancing the efficiency of therapy (7, 85). These mutations principally occur in the CpG sites, which are regions of the DNA molecule where the cytosine nucleotide is followed by a guanine nucleotide in the 5' to 3' direction. It was studied that the genome of GBM cells shows broad hypomethylation with specific areas of hypermethylation. The hypermethylation mostly occurs at the promoter CpG island of genes that are associated with tumor suppression, DNA repair, cell cycle regulation, apoptosis, invasion and migration (84). Overall, secondary GBMs showed a higher frequency of promoter methylation than primary GBMs; this can be caused principally for the *CpG* mutation sites that are more frequent in secondary (56% of the cases) than in primary (30% of the cases) GBM (30, 86). Loss of MGMT expression caused by methylation of promoter *CpG* islands was detected in 75% of secondary GBMs, more frequently than in primary GBMs (36%) (30), indicating that MGMT principally promotes methylation in secondary GBMs. The difference in frequency of *Mgmt* methylation between primary and secondary GBMs are clinically relevant because GBM patients with epigenetic silencing of the methylated *Mgmt* promoter are associated with loss of MGMT expression and diminished DNA-repair activity generating a greater benefit from adjuvant TMZ treatment (87).

Currently, MGMT is one of the most important DNA repair enzymes in GBM response, that removes the promutagenic methyl groups from the O-6-methylguanine adducts of guanine in the DNA molecule, and transfers the methyl group to an internal Cysteine (Cys145) residue in the enzyme preventing the G:C to A:T transitions (81, 83, 88). This effect of MGMT causes an increase in chemoresistance (88) neutralizing the cytotoxic effects of alkylating agents such as TMZ (30, 81, 89, 90). In GBM, promoter methylation of the gene encoding for MGMT is undoubtedly the genetic fingerprint with the highest impact on clinical practice. The *Mgmt* promoter hypermethylation is detected in approximately 32–72% of cases (35%−45% in malignant gliomas WHO grades III and IV and 80% of WHO grade II gliomas) (86, 87, 91, 92). In long-term survivors, the values are higher (74–83.3%) (93). One of the most important genes that promote the hypermethylation is the *Mgmt* gene, located at chromosome 10q26 (87, 88). Patients with GBM showed heterozygous deletion in the chromosome 10q26 (88), suggesting that the presence of an epigenetic lesion in DNA like that can suppress the hypermethylation of tumor genes. A correlation between the presence of *Tp53* mutations and *Mgmt* promoter methylation was found in GBM. Here a low-grade astrocytoma with *Mgmt* methylation was present in 92% of the *Tp53* mutations (30). Furthermore, G:C→A:T transition mutations at *CpG* sites were significantly more frequent in low-grade astrocytoma with *Mgmt* methylation (58%) than in those without (11%) (30). These results suggest that loss of MGMT expression due to promoter methylation frequently occurs at an early stage in the pathway leading to secondary GBMs and *Tp53* mutations at *CpG* sites in low-grade gliomas by exogenous or endogenous factors that produce DNA adducts at the O-6 position of guanine.

On the other hand, less than a half of the *Idh* wild-type of GBs have a hypermethylated *Mgmt* associated with the *CpG* island that depresses the MGMT expression and makes GBMs more sensitive to TMZ chemotherapy (85, 94). The methylation of the *Mgmt* promoter has been identifying as an important biomarker for GBM and is present in approximately 40% of the cases (95). The anatomic distribution of *Mgmt* promoter

methylated in GBM tumors is proposed to occur as part of a genetic signature that develops from lower-grade gliomas.

This transformation is thought to occur early in tumor development within glial cells in specific locations (96, 97) supporting the hypothesis that GBM development from neural stem cells (98) and the fact that many gliomas are contiguous with the posterior subventricular zone (99). Using the Analysis of Differential Involvement (ADIFFI) statistical mapping technique in a total of 358 patients with GBM, it was demonstrated that human GBMs occur in a high frequency contiguous with the posterior subventricular zone of the brain, *Mgmt* promoter methylated GBMs are lateralized to the left hemisphere, while *Mgmt* unmethylated GBMs are lateralized to the right hemisphere (84). Tumors closer to the left temporal lobe have a significantly longer overall survival compared with tumors occurring elsewhere, independent of treatment or *Mgmt* methylation status (99). Epigenetic silencing of the *Mgmt* gene by promoter methylation is associated with longer survival time and increased sensitivity to chemotherapeutic alkylating agents in GBM patients. However, patients with equivalent *Mgmt* promoter methylation status have variable prognoses and responses to treatment (100), suggesting that other factors are equally important in determining clinical outcome. We propose is that TNT mediated communication and spread of MGMT contributes to the clinical outcome.

Regulation of MGMT mRNA expression is related to favorable treatment response. *Mgmt* promoter methylation is as a powerful predictor of a survivor because hypermethylation of *Mgmt* is frequently expressed in long-term survivor patients (101). Although not all patients with methylated promoter have the same response to TMZ treatment, suggesting that methylated promoter is not the only factor involved in GBM treatment resistance (100). MicroRNA (miRNA) expression experiments classify and predict MGMT distribution in GBM samples based on mRNA expression profiles. A study in a cohort of 150 primary GBM showing that MGMT miRNAs were found to be differentially expressed between non-tumor brain tissue (100). Furthermore, an equivalent *Mgmt* promoter methylation, high- and low-risk patients have distinct prognoses, with the former showing a similar survival to GBM patients with unmethylated *Mgmt* promoters. It was found that high-risk patients with a methylated *Mgmt* promoter, who were treated with alkylating agents, had no survival advantage over low-risk patients (100).

Glioblastoma Metabolism: A Potential Mechanism of Heterogeneity Mediated by TNTs.

Tumor metabolism is based on two major points of cell behavior: (1) the specific sourcing of macromolecules of metabolites, and (2) the different cellular mechanism used to deal with different nutrients for either anabolic construction or catabolic breakdown. GBM metabolism offers new or supplementary targets for GBM therapy. Critical features of energy metabolism are related to mitochondrial genetics and apoptosis regulation in GBM. GBM functional processes are linked to mitochondrial regulation involving genomic and mitochondrial gene mutations, mitochondrial protein expression modifications and altered metabolic regulation. Mitochondria have a crucial role because they perform numerous important cellular functions: energy generation by synthesizing ATP via oxidative phosphorylation (OXPHOS), anabolic/catabolic reactions, metabolic regulation, signal

transduction, calcium homeostasis, reactive oxygen species (ROS) generation, redox control, and apoptosis. In the point of view of cancer metabolism mitochondria are indispensable for energy production and the survival of the cells, also are a crucial regulator of the apoptotic pathways. Warburg described that proliferating cancer cells preferentially convert glucose into lactate instead of pyruvate into the tricarboxylic (TCA) cycle of the mitochondria, even in presence of oxygen (102, 103). This process is known as aerobic glycolysis or Warburg effect.

The metabolic signatures of cancer cells are not responses to damaged mitochondria but result from oncogene-directed metabolic reprogramming required to support anabolic growth. Furthermore, mitochondrial DNA is a highly polymorphic molecule susceptible to a high mutational rate, which is caused by the lack of protective histones, proximity to the site of the production of (mutagenic) ROS and relatively limited DNA repair mechanisms. The high metabolic activity of cancer cells, impaired repair mechanisms, and increased genomic instability are typically susceptible to the accumulation of somatic DNA mutations including mtDNA mutations, which are also believed to contribute to cancer genesis and biology. Changes in mtDNA alter gene expression profiles and contribute to the compromised mitochondrial machinery of energy metabolism and apoptosis regulation. GBM tumor cells carry mtDNA mutations preferentially in the D-loop and protein coding regions and occur in the early stage of gliomagenesis (104).

Furthermore, the regulation of pro- and anti-apoptotic factors is deflected in all cancers. Upregulation of the antiapoptotic Bcl-2 and Bcl-2-like 2 (Bcl- XL) and downregulation of the proapoptotic Bcl-2-associated X protein (Bax) have been recurrently detected in GBM (105). Also, the energetic function of mitochondria in most malignantly transformed cells are related to the Warburg effect. It is based on mitochondrial impairment to oxidize glucose carbon to CO_2. While normal cells will largely undergo oxidative phosphorylation in the presence of glucose and oxygen, in many cancer cells the large proportion of glucose is diverted away from mitochondrial oxidation and into glycolysis and the production of lactate by lactate dehydrogenase (LDH) even in the presence of oxygen. GBSCs have been reported to have distinctly different metabolic phenotypes compared to more differentiated tumor cells, and appears to be able to easily switch between glycolytic and oxidative metabolism depending on the microenvironment (106). So, GBSC population maintains a distinct metabolic phenotype compared to the tumor bulk. Glucose uptake, glycolytic enzymes, lactate, and ATP production, are much higher in GBSCs compared to when they were differentiated, due to diminished metabolic contribution from mitochondrial oxidation (107). Anyway, metabolism and GBM correlation require a mention of isocitrate dehydrogenase 1/2 (*Idh1/2*) mutations. *IDH1/2* are responsible for catalyzing the oxidative decarboxylation of isocitrate into 2-oxoglutarate (or α-ketoglutarate, α-KG). α-KG is a key molecule in the Krebs cycle. It is nitrogen scavenger and a crucial precursor of glutamine and glutamate (108). It has a potent antioxidant and immune regulation function. Mutations in the *Idh1* and its homolog *Idh2* gene are very common in GBM. The loss of normal enzymatic function and the abnormal production of 2-hydroxyglutarate (2-HG) reduce the amount of α-KG (109). We propose that exchange of mitochondria within the tumor and TNTs contributes to metabolic signaling of GBM.

Tunneling nanotubes in Glioblastoma

Tumors are complex dynamic structures; cellular and molecular changes contribute to disease pathobiology. TNTs plays a key role in cancer pathogenesis, brain invasion, proliferation, and long-distance cell communication (110). Considering that the 10–20% of the cells in the tumor are malignant and may not be close enough to exchange cellular information through GJ (111), TNTs become a critical cellular communication mechanism for tumor evasion and chemotherapy resistance. TNTs are long cytoplasmic F-actin extensions of astrocytes and oligodendroglioma cells; their measures are 50–1,500 nm in length, 1 μm in width and 1.57 μm^2 of mean cross-sectional area (111–113). For example, TNTs connect 10–15% of Jurkat T cells in normal tissue culture conditions, and individual myeloid cells can support up to 75 nanotubes (114). There are different types of TNTs, an open-ended or connexin-containing protrusion (110, 115). GJs play a cooperative role in the communication system between the connected cells by TNTs. The presence of connexin 43 (Cx43) under HIV pathogenesis shows that the inhibition of GJ does not prevent TNT formation but interfere with the normal communication between TNT connected cells (116). TNTs are composed of Cx43. The functional role of Cx43 in astrocytoma progression of GBSC shows that Cx43 stabilizes the TNT communication. Furthermore, Cx43 deficiency results in reduced tumor size as observed by MRI and improved survival, also decreasing the radioprotective effect of TNTs in connected astrocytoma cells (113). For demonstrating tunneling microtubes (TMs) implication in therapy resistance, Weil and colleagues used surgical lesion experiments and implanted patient-derived GBM stem-like cells under a cranial window in mice, using *in vivo* 2-photon microscopy. They followed individual tumor regions and single glioma cells over extended periods. After the surgical removal of a cylindrical brain tissue volume colonized by GBSCs, GBM cells repopulated the lesioned area over time (56). This means that TNTs are involved in mediating the repopulation process. TNT-connected glioma cells are more resistant to the cytotoxic effects of TMZ chemotherapy and the microtube-connected astrocytoma cells were protected from cell death inflicted by radiotherapy (113).

In GBM, interactions and intercellular communications between malignant and non-malignant cells in the tumor microenvironment are tumor-promoting and critically to improve the understanding of the disease (117). Perivascular niche plays a crucial role in many aspects of brain tumor progression. GBM stem-like cells colonized the perivascular niche in significant numbers; it is possible to use them as a route for effective brain invasion. This was partly due to TNTs, followed the perivascular room of the dense brain microcapillary network as a leading track. A subgroup of GBSCs in a perivascular position showed long-term latency and targeting this subpopulation of glioma cells emerges as an important task for the development of novel therapies, since existing treatment modalities fall short of controlling these cells (118). It has been widely demonstrated that TNTs allow the bi- and unidirectional transfer of cargoes, including protein, mRNA, and organelles such as mitochondria and endoplasmic reticulum (ER) between the connected cells (110, 112, 113, 119–122).

Nevertheless, the mechanisms of selectivity, transport, and delivery are still unknown. The transfer of cargoes is a fast process, taking account that TNTs lifetime is less than 60

minutes (113). Several laboratories demonstrate that TNT formation is controlled by *p53*, epidermal growth factor receptor (*Egfr*), Akt, phosphoinositide-3-kinase (*PI3K*) and *mTOR*. *p53* activation or *Egfr* or *Akt/PI3K/mTOR* induces M-Sec overexpression, which can trigger F-actin polymerization and contributes to TNT development from the initiating cell membrane (123). In addition to TNTs formation has been studied in cancer cells, neurons, immune cells (B, T, NK cells, neutrophils, monocyte, macrophages and dendritic cells, monocytes), endothelial cells and stem cells (55, 112, 115, 124–126). It has been demonstrated that TNT number increases when cells are under stress or pathogenic context. For example, it was demonstrated that the number of TNTs formation between astrocytes and C6 glioma cells was increased in the presence of H_2O_2 (127). Also, our preliminary data show that TNT radiation and TMZ treatment are stressful handlings that induce TNT formation.

Furthermore, in GBM, TNT formation is highly influenced by tumor type and grade, with a marked positive correlation of TNTs length and unfavorable prognosis. This data suggests that cells under pathological conditions (cancer or infectious diseases) can communicate and cooperate in a complex but ordered manner. Thus, TNTs formation and function will open new therapies for the treatment of different diseases. Our group proposes that TNTs proliferate due to radiation and TMZ treatment and help tumor invasion and survival (55). We also suggest that the spread of MGMT protein into cells with insufficient or lacking MGMT occurs via TNTs to adapt the tumor to treatments and increase reccurence (see Figure 2). These mechanisms are novel. Thus, we propose that TNTs are a novel route by which MGMT and other tumor protective molecules could be transfered between non-susceptible cells to treat tumor to susceptible tumor cells preventing tumor cell death. Interestingly, TNTs are minimally expressed under physiological conditions and are only induced in cancer. Thus, TNTs are an exciting therapeutic target.

The transfer of mitochondria via TNT cause alterations in cell metabolism and consequently affect cancer cell proliferation, differentiation, apoptosis, and response to therapy, through the intervention of several metabolic pathways (55, 128). Mesenchymal stem cells (MSCs) form TNTs and transfer mitochondria to target cells (including cardiomyocytes, endothelial cells, epithelial cells, macrophages, and cancer cells) under conditions of stress or injury, leading to modifications of the functional properties of these cells energy metabolism and functions (121, 129, 130). This process was studied between MSCs, neural cells and astrocytes *in vitro* and rat brain *in vivo* (131). Specifically, healthy or mutated mitochondrial DNA (mtDNA) can be shared between cells, and consequently affects the metabolism and the correct function of the affected cell. It has been reported that the horizontal transfer of mtDNA from host cells in the tumor microenvironment to tumor cells with a compromised respiratory function re-establishes respiration and increases tumor-initiating efficacy. Showing that tumor cells without mtDNA display delayed tumor growth, and that tumor formation is associated with the acquisition of mtDNA from host cells (132). Furthermore, it had been shown that mtDNA is acquired by transfer of whole mitochondria from stromal cells of the syngeneic mice, resulting in the recovery of respiration in tumor cells with damaged mtDNA and efficient tumor formation (133).

Mitochondria exchange between neural and MSCs improves the protective abilities for the affected cells (131). The intercellular exchange of mitochondria, via TNTs has been proposed as a mechanism for restoring damaged cells and may provide complementary effects on the mitochondria in the cells affected by ultraviolet radiation. The interaction between apoptotic and healthy cells connected by TNTs shows that in pheochromocytoma cells (PC12) treated with ultraviolet light (UV) were rescued when they were cocultured with untreated PC12 cells (134). Furthermore, the decrease of TNTs blocked the intercellular transfer of mitochondria and inhibited the rescue effect (134). Additionally, the TNT formation between stromal (MSCs and endothelial cells, ECs) and cancer cells, showed that intercellular transfers of cytoplasmic content occurred similarly between cancer cells and MSCs or ECs, but the exchange of mitochondria occurred preferentially between ECs and cancer cells. Cancer cells acquiring mitochondria displayed chemoresistance (128). TNTs may help drug-sensitive cancer cells to acquire survival signals from drug-insensitive cells and escape death during cancer treatment, suggesting TNTs as a target for the development of new cancer therapies.

Drugs and Treatments

Currently, the treatment for GBM corresponds to surgical resection of the tumor, where the patient waits approximately four weeks for the craniotomy wound to heal before starting the therapy (135). The post-surgery process receives radiotherapy irradiation of 2 Gy given 5 days per week for 6 weeks, for a total of 60 Gy (13, 136) plus continuous daily TMZ, 75 mg per square meter of body-surface area per day, 7 days per week from the first to the last day of radiotherapy, followed by six cycles of adjuvant TMZ, 150 to 200 mg per square meter for 5 days during each 28-day cycle (13). This treatment prolongs patient survival and reduces the risk of death by 37% (13). Despite treatment, recurrences are observed within 6–7 months and occur in around 90% of the cases (137, 138), may be due to the tumor adaptation, it has been found that the reappearance of GBM arise at the resection margin, wherein the highest doses are delivered and is caused by the residual GBM cells left in the surgical margins, in the peritumoral tissue between 2 cm from the tumor edge or infiltrating the normal brain parenchyma (138–140). These residual cells subsequently become exposed to standard and experimental therapy, although their study and characterization open new research lines of therapy. GBSCs have been demonstrated as being responsible for the tumor recurrence. A small population of GBSCs derived from both peritumoral tissue and GBM shows significant differences between GBM and peritumoral tissue regarding proliferation, ultrastructural peculiarities and, at a lower extent, stemness profile (139). The residual GBM cells are difficult to image and the maximum dose of radiotherapy cannot destroy these cells without a specific target. Chemotherapy can eliminate these cells, but not completely, an *in vitro* drug and irradiation (5 Gy), plus concomitant treatment with temozolomide (500 μM), lomustine (380 μM), and combinations, shows that in 64% of the cases GBM periphery cells responded dissimilar from the corresponding center cells (140). Finding new treatments to eliminate the residual GBM cells is a major challenge.

Other factors affect the progress of therapies including invasive tumor growth in a vital organ limiting the utility of local therapy, protection of tumor cells by the BBB, intrinsic resistance to the induction of cell death and lack of dependence on single, targetable

oncogenic pathways (141). Predictive molecular markers are commonly tested as part of the routine clinical interrogation of GBM patients including *Mgmt*, *Idh*, *Egfr*, *Vegf*, *Tp53*, phosphatase and tensin homolog (*Pten*), *p16INK4a* gene, phospholipid metabolites, cancer stem cells, and recently also imaging biomarkers. Meta-analyses are used to augment the validity of potential prognostic biomarkers in GBM, but significant limitations are due to GBM novel nature and incomplete understanding of GBM biology (142). Several specific biomarkers need to be investigated for a distinct prognosis, for trying to personalized therapeutic approaches and for contributing to the development of a new generation of anti-GBM therapies (143). New progress in GBM therapy combines the current standard-of-care treatment and immunotherapies or alternating electric fields therapy. CNS is a privileged immune organ, but microglia are the major antigen-presenting cells in the brain tumor microenvironment (144), which could be a strategical target for immunotherapy. Escape immune system surveillance is a critical feature for GBM, and several immune suppressive mechanisms are utilized in the setting of GBM to prevent its immune detection and eradication.

The increased signal transducer initially drives immunosuppression, and activator of transcription 3 (*STAT3*) expression that induces cell secrete immunosuppressive factors production such as TGFβ−2, prostaglandins (PG), interleukin (IL)-1, IL-10 and fibrinogen-like protein 2 (FGL2). Regulatory T cells (Tregs) are the major responsible cells that suppress immune responses by secreting cytokines (TGF-β and IL-10), by cell-to-cell mediated contact and by cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) activation (145). In GBM, macrophages have increased levels of programmed cell death 1 ligand 1 (PD-L1) that binds to and activates PD1 immune-checkpoint receptor restricting cytotoxic T cell activity. GBM vaccine therapy relies on dendritic cells (DC)-mediated presentation of GBM-associated peptides, multi-peptides, cell-activating adjuvants, antigens, or epitope derived from tumor lysates to T cells. Many clinical trials are ongoing to evaluate the efficacy of these vaccines. Genetic engineering uses oncolytic viral therapy to create viruses that selectively infect or replicate in tumor cells. The resulting tumor cell lysis not only kills the infected tumor cells directly but can also activate the immunogenic tumor cell death pathway that can stimulate antigen presentation and adaptive immune response. Also, others immunotherapy approaches are antibodies that block the inhibitory immune-checkpoint proteins (PD-1 and CTLA-4) or engineered chimeric antigen receptors, but it seems more efficacious to combine the traditional standard of care with these innovative immunotherapy strategies (141). However, the effectivity of these treatments in GBM is unclear.

Artificial Intelligence to Understand Glioblastoma Heterogeneity and the Role of TNTs.

Currently, advances in computational and data science have been increasing, with the purpose to find more comprehensive and coherent strategies to improve health care and medicine. Artificial intelligence (AI) and machine learning (ML) algorithms in cancer research are a powerful tool to increase the speed in the efficiency to improve the diagnoses and design new therapies, drugs, and treatments. All these new technologies have the main focus to cure or increase life expectancy in cancer patients.

In cancer image analyses, AI has a successful domain. The radiologist uses a collection of images (X-ray, tomography, magnetic resonance imaging (MRI) and positron-emission tomography) to screening and diagnosis cancer (146). Furthermore, histopathological assessment to classifying cancer and identifying metastasis also apply ML and AI to analyses the microscopy results and improve the accuracy in the analysis. These studies employed the transfer learning technique to establish neural network connections of thousands of images database (146). To classify and identify the cancer diagnoses in GBM radionics research help to predict disease prognosis, they are providing beneficial information for personalized treatment from a variety of imaging features extracted from multiple MRI. The Early diagnosis of cancer is a critical point in term of life expectation and treatment. The computational methodologies (AI or ML) to predict early stages or the detection of tumor cells are crucial. However, metastatic tumor cells are exceedingly difficult to detect from blood or biopsy samples. But It is reported that three ML algorithm combined can analyze the data from microscopy images quickly and quantify the cell morphology for instant real-time feedback can certainly contribute to early cancer diagnosis (147). The imaging analysis not only is useful for early detection but also the prediction of survival.

The prediction results for both 2-class (short and long) and 3-class (short, medium, and long) survival groups were 98.7% and 88.95% respectively (148). In GBM, the methylation status of the promoter of the MGMT gene impacts the efficacy and sensitivity of the TMZ treatment and consequently affects patient survival. Microscopic genetic changes may manifest as macroscopic morphological changes in the brain tumors that can be detected using MRI (149). A neural network analysis of brain MRI scans of GBM patients collected from The Cancer Imaging Archive (TCIA) combined with methylation data from The Cancer Genome Atlas (TCGA) to predict the methylation state of the MGMT regulatory regions in these patients. The results with 67% on the validation data suggest the existence of MRI features that may complement existing markers for GBM patient stratification and prognosis (149). Besides the good results in the application of the AI methodologies to the imaging analysis, there are limitations that need to be told in considerations such as the different imaging platforms, the protocols and parameter used to get the images, the criteria to classify the patients and the demographic and treatment information of the patients (150, 151). There is extra work in the direction of strengthening the ML and AI classification models based on imaging data for reliable and clinically meaningful prediction of the assessed molecular characteristics in patients diagnosed GBM.

A large amount of data and information as OMICs data (metabolomic, proteomic, lipidomic, genomic and transcriptomic), that are associated with the response of patient-specific phenotypes to drug therapy at the molecular level in cancer making impossible the unification of this data for subsequent analysis without AI or ML methodologies to find metabolic pathways related with the evolution of the tumor microenvironment, the progression of the disease in the patient, the type of cancer. The development of different cancer database as TCGA Research Network has provided and analyzed human tumors with the purpose to find genomic alterations in DNA, RNA, protein and epigenetic level, over 11,00 tumors from 33 types of cancer are present in this database (152). All these enormous amounts of information provide a major opportunity to develop an integrated methodology

that involves statistical analysis and computational approaches as AI and ML to develop effective therapies for different cancer type (153). For example, an ML method is capable of identifying stemness or the potential self-renewal and differentiation from the origin cell, in a single-cell pattern of intra-tumor molecular heterogeneity (154). In GBM, a multigene predictor was developed, using GBM microarray data from 4 independent data sets is capable of identifying 9-gene sets, as an independent predictor of outcome in GBM survival(155). Furthermore, TCGA was used to train accurate predictors for NF1 inactivation; this gene is an important regulator of the oncogene RAS and is inactivated frequently in GBM (156). With the current database and improved classification, ML and AI models will translate into clinically relevant predictions that will guide GBM therapy. Now critical markers of TNTs can be added to these analyses to identify their role in the pathogenesis of GMB as well as develop new treatment to cure GBM.

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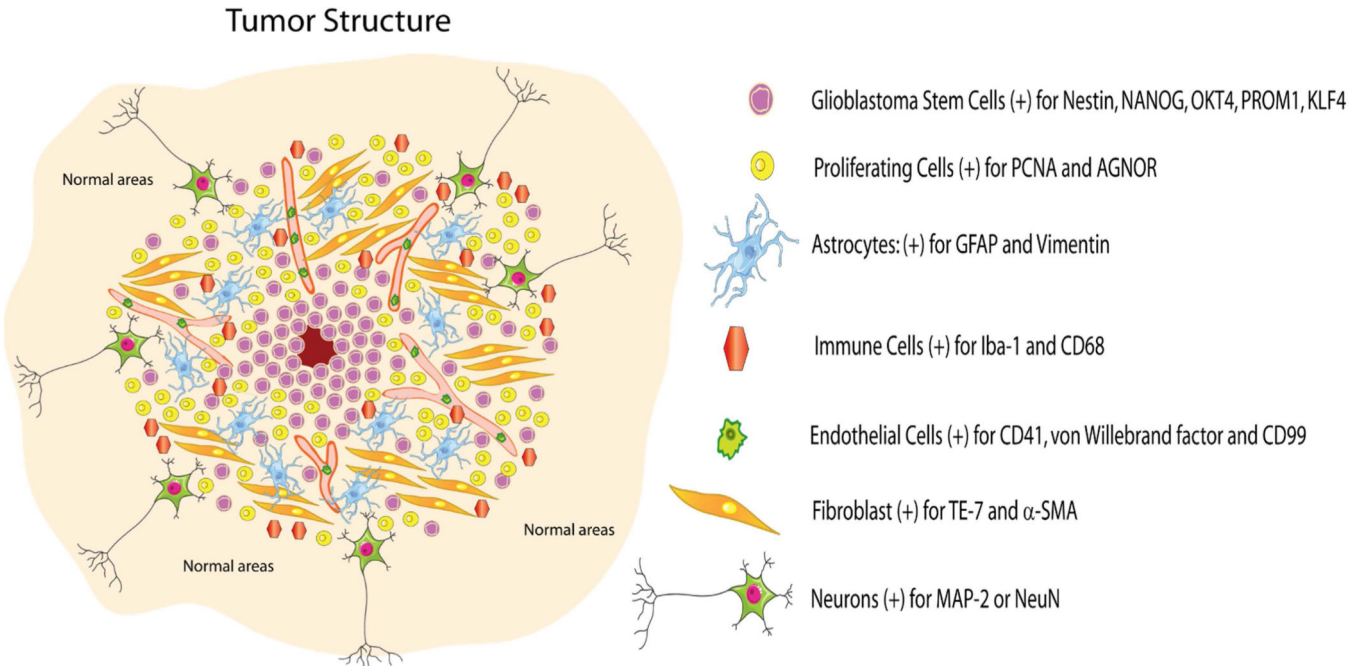

Figure 1:

Glioblastoma tumor structure and heterogeneity. The tumor is comprised of several cell types including GBSC positive for Nestin, NANOG, OKT4, PROM1 and KLF4; proliferating cells positive for PCNA and AGNOR; astrocytes positive for GFAP and Vimentin; Immune cells positive for Iba-1, CD68 and CD3; endothelial cells positive for CD31m von Willebrand factors and CD99; fibroblast positive for TE-7 and SMA and neurons positive for MAP-2 or neuN. Most of these areas are repetitive and with multiple mutations. Thus, the heterogeneity of the tumor is extremely high

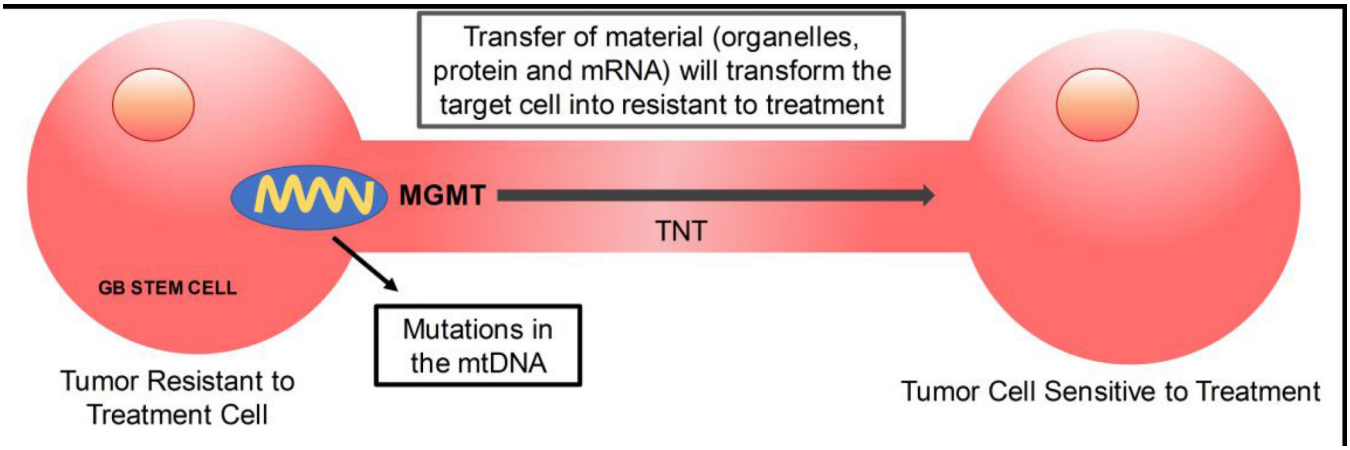

Figure 2:

Transfer of MGMT and altered mitochondria (with mtDNA mutations) from Glioblastoma tumor resistant to treatment of cancer stem cells to glioblastoma tumor sensitive to treatment stem cells, via tunneling nanotubes (TNTs).