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TOPIC REVIEW

The challenges associated with molecular targeted therapies for glioblastoma

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Abstract Glioblastoma (GBM) is the most aggressive malignant brain tumor in adults. Improvements in the treatment of GBM have remained static since the advent of the standard therapy which includes radiation with concurrent and adjuvant temozolomide treatment. Developing treatment and diagnostic or companion biomarker combinations is transforming the way we treat numerous cancers. However, can this emerging paradigm be also effective for GBM? Can GBM be treated the same way as other cancers? Here we review the challenges for a personalized molecular targeted therapeutic approach in GBM. The specific challenges for establishing a personalized molecular targeted medicine program for GBM patients include overcoming the blood brain barrier, unravelling the intra- and inter-heterogeneity that exists and the importance of developing more relevant animal models that recapitulate a patient's GBM tumor.

Keywords Personalized medicine · Glioblastoma · Biomarkers · Chemotherapy

Introduction

The survival trends for patients diagnosed with glioblastoma (GBM) have remained largely static, reflecting a lack of improvement in the therapeutic options for patients. Prognosis is poor for most patients diagnosed with GBM and less than 5 % of newly diagnosed GBM survive more than 5 years. GBM are highly refractory to treatment with local tumor recurrence occurring 2–3 cm from the original resection cavity (the area exposed to radiation treatment) frequently observed. Relapsed GBMs are difficult to manage with a median survival of only a few months after recurrence [1]. Increasingly, the development of novel therapies involve defining drug-diagnostic combinations where the presence of a molecular target or marker identifies patients who are most likely to respond to a specific therapy. This model of developing treatment and diagnostic/companion biomarker combinations is the emerging paradigm for novel drug and diagnostic development [2–4] with a recent example being the use of BRAF inhibitors, which target a specific activating mutation of BRAF (V600E) in melanoma [5]. GBM is characterized by inter- and intra-patient genomic and histopathological diversity, arising from the complex dynamics that underpin its development. Given this, a single "bullet" approach is unrealistic.

Temozolomide (TMZ) chemotherapy improves the survival of patients with GBM by a few months when used in combination with radiation therapy [6, 7]. However, GBMs with methylation and suppression of the *O-6-Methylguanine DNA methyltransferase* (*MGMT*) promoter region of the gene are more sensitive to TMZ [7, 8]. Rindopepimut (Rintega®) received Breakthrough Therapy Designation from the FDA to treat epidermal growth factor receptor variant III deletion mutation (EGFRvIII) positive GBM [9]. Results from the ACT III study showed a median overall survival of 21.8 months, and 36-month overall survival was 26 % [9]. In the future, as more chemotherapeutic agents with similar efficacy are developed based on molecular alterations, it may be possible to design clinical trials assessing the differential sensitivities of patients with different molecular signatures and alterations to chemotherapy.

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The aim of this paper is to discuss the challenges that are possibly faced in the practical application of an individualized medicine approach for GBM patients, from molecular challenges to translating the results to the bedside.

Molecular subtypes of GBM

Breakthroughs in human genomics have led to exciting prospects in the field of cancer therapy. The profiling of the human genome has given scientists and researchers an insight into the possibilities of the DNA being the root source of a person's susceptibility to a certain disease or condition. This discovery has led to an era of—omics technology, where treatment is focused on molecular targets specific to a patient's genomic data. GBM was the first malignancy to be studied by The Cancer Genome Atlas (TCGA) Research Network [10]. The key aims of TCGA are "to identify the changes in each cancer's genome and understand how these changes interact to drive the disease, thereby laying the foundation for improved cancer prevention, early detection and treatment", with priority afforded to cancers associated with the highest disease burden. TCGA GBM project, conducted in two phases, has provided a comprehensive genome-wide map of the genetic, epigenetic and transcriptomic changes, as well as proteomic changes, in over 500 GBM samples [10, 11]. This led to the identification of four distinct molecular subtypes of GBM on the basis of signatory gene expression profiles, namely classical, mesenchymal, neural, and proneural. These have also been associated with particular genetic alterations [12]. The Classical subtype is associated with an astrocytic expression profile, with frequent *EGFR* amplification, concomitant chromosome 7 amplification and chromosome 10 loss, and focal deletions of 9p encompassing *CDKN2A*. Although *TP53* mutations are common in GBM, the Classical subtype lacks these. The mesenchymal subtype is typified by the expression of mesenchymal markers, with frequent deletions or mutations of the *NF1* and *PTEN* genes. The neural subtype exhibits expression of neuronal markers and displays various mutations and copy number alterations including amplification of *EGFR* and deletion of *PTEN*. The proneural subtype exhibits an oligodendrocytic expression signature and is characterized by focal amplifications of the chromosome 4q12 region containing the oligodendrocytic development gene *PDGFRA*, or mutations of the isocitrate dehydrogenase 1 gene, *IDH1*.

In terms of prognosis, no difference was found between the classical, mesenchymal and neural subtypes. However, the Proneural subtype was associated with younger age and prolonged survival time [12]. This has since been attributed to the fraction of cases that have somatic *IDH1* mutations. *IDH1* wild-type tumors within the Proneural subtype did not show this survival advantage [13]. *IDH1* mutations have consistently been associated with improved overall survival in patients with glioma [14], primarily because *IDH1* mutations occur most frequently in grade II-III gliomas and secondary glioblastoma and are associated with younger age [15]. Examinations of response to radiation or chemotherapy in randomized trials of high-grade astrocytoma have not found a therapeutic interaction between adjuvant treatment and IDH1 genotype [16]. However, *IDH1* mutation status has been associated with therapeutic benefit from maximal surgical resection [17].

Targeted therapy

Targeted therapy has shown successful results in other cancer groups including breast cancer, non-small cell lung carcinoma (NSCLC), bowel cancer and melanoma. In breast cancer, the discovery of trastuzumab as an adjuvant therapy revealed promising results in human epidermal growth factor 2 (HER2) positive patients. The addition of trastuzumab in the treatment program proved a substantial increase in the survival of HER2 positive breast cancer patients [18, 19]. NSCLC patients with *EGFR* mutations are known to respond better with EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib. However, EGFR TKIs are avoided in NSCLC patients with *KRAS* mutations, which cause innate or acquired resistance to EGFR TKIs [20, 21]. Similarly, patients diagnosed with colorectal cancer with *KRAS* mutations are not given EGFR TKIs for the same reason. Other inhibitors are given instead to these patients to target different molecules such as sorafenib of which inhibit the RAF kinase family (*ARAF, BRAF, CRAF*) and are downstream molecules of RAS [22]. Mutations in the *BRAF* gene are used as markers for patients with metastatic malignant melanoma. BRAF positive metastatic malignant melanoma benefit from BRAF inhibitors such as vemurafenib [23].

There is significant progress in our understanding of the molecular biology of GBM, which has resulted in new advancements in targeted therapy. Two of the most prominently studied antigens are *EGFR* and its tumor specific genetic deletion variant III (*EGFRvIII*). *EGFR* is amplified and/or mutated in up to 60 % of GBM. Tumor growth and survival are promoted by *EGFR* through consistent activation of signaling networks and metabolic reprogramming [24]. As mentioned earlier, the injectable peptide vaccine, rindopepimut has shown significant efficacy in tumors positive for *EGFRvIII*. The seminal study by Sampson and colleagues showed significantly increased survival when compared with a patient history matched group of patients and that this survival gain was associated to increased titer of anti-EGFRvIII antibodies in the serum [25]. In addition, tissue collection

of 9 of 11 patients undergoing second surgery showed loss of EGFRvIII expression after treatment, suggesting that the immune cells remove EGFRvIII positive cells [25]. The survival benefits of rindopepimut treatment have been confirmed in the ACT III phase II study, however this was not a randomized study [9]. The clinical benefit of rindopepimut will be clarified with the ongoing randomized phase III study, ACTIV.

A monoclonal antibody drug conjugate (ADC), ABT-414, has shown significant efficacy against tumors expressing amplified *EGFR*. The results of the Phase I trial were reported at the Society of Neuro-oncology (SNO) meeting in 2014. Four of 12 patients (33 %) achieved an objective response, including two who achieved complete responses. A randomized phase II trial of ABT-414 has been initiated.

Erlotinib has gained regulatory approval to treat *EGFR* mutant lung cancer, however the results for the treatment of *EGFR* mutant GBM have been disappointing. Several clinical trials have tested erlotinib in combination with radiotherapy and TMZ, with sirolimus and with bevacizumab [26–29]. However, all trials showed negative results. The specific location of the mutations is important. The majority of the mutations in *EGFR* in GBM occur in the extracellular domain (ED) in contrast to mutations in lung cancer, which occur in the kinase domain (KD) [30]. Inhibitors like erlotinib target the active kinase conformation. Second-generation EGFR inhibitors such as dacomitinib are beginning to show promise, however much of this data is limited to pre-clinical models.

There are many other target-therapeutic combinations in GBM that warrant further exploration. Approximately 2–3 % of all GBM harbour mutations or activating gene rearrangements in BRAF. It would be interesting to determine the response of these mutants to sorafenib. At the recent SNO meeting at San Antonio, 2015, the Adaptive Global Innovative Learning Environment (AGILE) trial was announced. Starting in 2016, this adaptive trial design will test patient tissue for biomarkers and assign treatment based on molecular biology.

Challenges in GBM

Targeting specific molecular aberrations seem to be the most logical and efficient approach for cancer treatment for GBM patients. However, challenges can be observed that may hinder a GBM patient's way to recovery. The most significant challenges include: inter- and intra-tumor heterogeneity and the blood brain barrier (BBB). Both of these factors may possibly contribute to chemotherapy resistance in GBM patients and complicate treatment response and prognosis [31, 32]. Other factors that should be considered include tumor sampling times, limited therapeutic armamentarium, and low availability of pre-clinical models to be used for testing novel and repurposed drugs prior to giving these to the patient.

Blood brain barrier

Overcoming the blood brain barrier (BBB) has been a long-standing challenge in the treatment of GBM. The BBB is formed by a neurovascular unit comprising endothelial cells enforced by astrocytes and pericytes. This forms an extensive network of capillaries that prevent various substances from the blood stream from entering into the brain [33, 34]. The major factors affecting penetration have been discussed in several reviews and include the substance's molecular weight, lipid solubility, and polarity [33–37]. Additionally, the presence of multiple transport proteins in the endothelial lining of the BBB's vascular component is another major factor considered that inhibits the drug from reaching the target tumor tissue [33, 34, 38]. A few examples of drugs that were shown to have limited efficacy for GBM include pazopanib, paclitaxel and doxorubicin. Pazopanib, an oral VEGF inhibitor that has been observed to be effective in renal cell carcinoma, breast and lung cancer cannot be given to GBM patients due to this barrier and efflux mechanism [39, 40]. Similar to pazopanib, paclitaxel and doxorubicin are other chemotherapeutic drugs more commonly used in other cancer groups that has been investigated for efficacy in the treatment of GBM but had poor results due to the presence of multi drug resistant proteins such as the p-glycoprotein [41, 42].

Different drug delivery methods are being investigated to bypass the BBB. Osmotic BBB disruption (BBBD) makes use of chemicals such as mannitol or bradykinin to disrupt the integrity of the tight junctions in the BBB. Two retrospective studies demonstrated survival benefit in patients receiving chemotherapy with BBB modification by intracarotid and intravertebral artery infusion of mannitol [43, 44]. This disruption increases the spaces in between the tight junction thereby increasing drug permeability [33, 34, 45]. Unfortunately, this method has limited usefulness due to toxicity and complexity of the procedure. Another method that result in opening of the tight junctions between the endothelial cells is the transcranial delivery of low frequency ultrasound waves (focused ultrasound [FUS]). Pre-clinically, enhanced brain penetration of carmustine (BCNU) was observed in rat models using a combination of microbubbles (MBs) and focused ultrasound (FUS) [46]. There have been no reports of this method used in patients. Convection-enhanced delivery (CED) is a localized delivery strategy involving continuous positive-pressure infusion of a solute containing a therapeutic agent [47]. This method has been used for the delivery of therapeutic proteins, oligonucleotides, liposomes and viral mediated

therapies [48]. CED has feasibility issues including technical expertise and the characteristics of the drug used. Catheter placement effects drug distribution and can also influence adverse effects such as chemical meningitis. Osmolarity, pH, ionic composition and drug solubility can also influence drug distribution by CED. In addition, the effect of CED on infiltrating glioma cells is questionable. The value of CED remains to be validated by a successful clinical trial.

A major mechanism by which the BBB limits drug delivery to the brain is the active efflux transport of molecules from the capillary endothelial cells. There are numerous active efflux transporters, including P-glycoprotein (P-gp), multidrug resistance proteins (e.g. MRP4) and breast cancer resistance protein (BCRP). Some methods take advantage of the presence of multi-drug efflux transporters at the BBB. Drugs, such as elacridar and tariquidar, have been developed to inhibit the function of multi-drug efflux transporters to increase drug influx through the BBB [33, 34]. In a very recent mouse study examining the efficacy of the CDK4/6 inhibitor, palbociclib, significantly higher levels of the drug were detected in the brain tumor when palbociclib was combined with elacridar [49].

Another approach plays on receptor-mediated transport mechanism where a 'shuttling factor' is coupled with the drug and goes through the BBB by targeting specific receptors such as insulin or transferrin [33, 50]. Alternatively, nanoparticles has likewise been used as drug vehicles to increase the chances of drug molecules, such as doxorubicin and paclitaxel, by-passing this barrier [51]. Circumvention of the BBB by directly administering the drug into the brain or tumor parenchyma through the use of intraventricular/intracavitary systems or polymer wafers has also been clinically investigated. An example of this approach is the use of carmustine (Gliadel) wafers. Gliadel wafers permit a slow release of carmustine (BCNU) after placement in the surgical cavity of high grade glioma. Despite significant survival benefits reported in Phase III studies, uptake to use gliadel wafers has been poor [52]. This may be due to the lack of phase III evidence that gliadel adds benefit to the current standard treatment; high infection rates, high cost of treatment and the fact that gliadel wafer placement frequently represents an exclusion criterion for recruitment of patients into further clinical trials.

Overcoming the physiological barriers with novel drug and diet options

Although numerous studies have been conducted to find treatment for GBM, only a handful of drugs have been FDA-approved. The development of new drugs could involve very lengthy processes and massive costs. On an average, a single drug takes 15 years and approximately US$800 million before a single drug is approved for marketing [53–55]. Repurposing drugs is being explored to compensate for the limited availability of drugs used for treatment of, not only, GBM but in other cancer groups as well [54]. Such in the case of the use of anti-fungal drugs as treatment for breast cancer and prostate cancer. Clotrimazole is being investigated in vitro for its effect on cell proliferation, viability and glycolysis in human-derived breast cancer cell lines [56]. Itraconazole, on the other hand, was recently investigated in a phase 2 clinical trial for castration-resistant prostate cancer (CRPC) [53]. Additionally, ibudilast, a drug that has been previously marketed for the treatment of asthma, is being investigated for its effects in combination with TMZ for the treatment of MGMT-unmethylated GBM [57]. Metformin is an oral anti-diabetic drug gaining much interest in the treatment of GBM. Metformin potentiates the pro-apoptotic effect of TMZ via the activation of 5′-adenosine monophosphate (AMPK). This enzyme plays a role in cellular energy homoeostasis, acting as a metabolic master switch and hence regulating several intracellular systems, including the inhibition of the mTOR pathway [58]. Hydroxychloroquine (HCQ), an autophagy inhibitor, has been shown to potentiate the effects of DNA damaging agents such as radiotherapy. Rosenfeld and colleagues tested the effect of HCQ in combination with RT and TMZ in a phase I trial design followed by a non-comparative phase II trial design [59]. No significant improvement in overall survival was observed.

Other non-conservative treatment options include the use of devices and diet. Recent Phase III data presented by Stupp and colleagues at the ASCO meeting in June 2015 have shown a significant survival benefit for the use of the Novo-TTF-100A (Optune^{TM}) device. The Optune device generates tumor treating fields directly to the patient's scalp and acts as an anti-mitotic therapy for GBM. The device gained FDA approval for its use however it is unavailable in many countries, including Australia and the cost of treatment is extremely high. Dietary options such as a restrictive ketogenic diet (KD) to result in glucose deprivation have been explored. A recent study developed a supplemented high-fat low-carbohydrate (sHFLC) diet and showed that this diet was able to reduce glucose in vitro and inhibited proliferation and tumor stem cell expansion [60]. Survival was extended in an orthotopic xenograft model.

Tumor heterogeneity

A critical problem with large cohort studies such as TCGA, is that single-tumor sampling leads to significant sampling

biases. Fluorescence in situ hybridizaton (FISH) analyses of sections of GBM revealed cells with mutations to *EGFR* and *PDGFR* co-existing within the same GBM [61]. Therefore, a single agent targeting the *EGFR* mutation may show limited efficacy in these tumor types because it fails to target the *PDGFR* gene aberration [62]. The discovery of cells with different driver mutations side by side within a single tumor suggests that targeting a single mutation may be an ineffective strategy in GBMs. In a study by Watts and colleagues, 38 fragments from 9 patients with GBM were sampled and genome-wide somatic copy number levels were measured [63]. Although the fragments from the same patient shared a common gene profile, indicating the clonal origin of the tumor, they displayed a striking variety of copy number alterations that were present in only a subset of fragments, indicating clonal evolution [63]. In addition using gene expression arrays, they found that in 6 out of 10 cases the fragments from the same tumor mass were classified into at least 2 different GBM subgroups (mesenchymal, neural, classical or proneural) [63]. This indicates that tumor clones with different phenotypic profiles coexist within the same malignancy. Yachida et al. demonstrated heterogeneity in pancreatic cancer using whole exome sequencing and copy number analysis of samples obtained from different anatomical regions of a pancreatic cancer [64]. This study showed that clonal tumor populations present in the primary tumor give rise to metastatic disease in a branched evolutionary pattern, with progressor mutations common to metastatic sites and within regionally separated subclones of the primary lesion [64].

Sampling times

Targeted therapy is typically utilized as a salvage therapy for glioblastoma patients when they relapse. Not all patients are suitable candidates for a second round of surgery. When it comes to biomarker analysis, the only tissue available for testing is typically the specimen used for the primary diagnosis. The genomic road leading to recurrence in GBM is not well understood. Most likely, after therapy, the surviving population may not be a single resistant cancer clone, but rather a heterogeneous population of malignant cells with genetic aberrations that allow them to survive the initial treatment. Shah et al., described the genome of a metastatic obular breast cancer with 19 non-synonymous mutations present in the metastatic lesion that were not present in the primary cancer diagnosed 9 years previously, illustrating the temporal dynamics of intra-tumor heterogeneity [65]. Clonal evolution, driven by genomic instability, underlies the development of metastatic pancreatic cancer [66]. A recent study by Verhaak examined the genomic events in pre- and post-treatment GBM pairs [67]. Through longitudinal comparisons of tumor samples before and after treatment, *TP53*-mutated tumors showed a further increase in clonal complexity at the time of relapse, whereas *TP53* wildtype recurrences appeared to have gone through a bottleneck, which resulted in relatively monoclonal recurrent tumors [67]. *TP53* mutations have been associated with an increased frequency of double-strand breaks and chromothripsis in medulloblastoma [68]. The apoptosis negating properties of *TP53* DNA binding domain mutations may result in an increased tolerance for acquiring and sustaining single nucleotide polymorphisms (SNPs) [67].

A potential strategy to overcome this issue of tumor sampling would be to re-biopsy the tumor at the time of progression and perform the molecular profiling on the recurrent sample. This approach may not be feasible for all patients, particularly when the tumor is located in eloquent areas of the brain. Measuring circulating tumor cell DNA (ctDNA) may be another viable and minimally invasive clinical option for GBM patients allowing clinicians to identify potentially druggable molecular alterations driving recurrence. These "liquid biopsies" can be collected from several sources including blood, urine and the CSF. As a result of cell death or active secretion, tumor cells can release small pieces of their DNA and/or RNA into the bloodstream or CSF and are relatively stable. Multiple molecular alterations including loss of heterozygosity of 1p and 19q, *IDH1* and *EGFRvIII* as well as the methylation status of promoters of *MGMT*, *PTEN* and *CDKN2A* have been detected [69–71].

Pre-clinical models

Solid and robust pre-clinical data is required to advance clinical trials for GBM patients. In this era of 'omics', there is a unique opportunity to use genomic profiling to identify biomarkers to treatments. Dynamic biobanking is becoming more frequent at large Neurosurgical centres. That is, at the time of patient surgery, the tumor is collected. Rather than just storing the piece of tissue in paraffin or cryopreservation, cells are dissociated from the tumor and grown in culture and/or immediately surgically implanted into the brains of immune-compromised mice. We appreciate now that more traditional, serum-based cell lines do not allow for recapitulation of patient tumor physiology and many of these cell lines cannot form tumor in vivo [72]. Cells grown as neurospheres or monolayers on a laminin-coated surface in a serum free environment with growth factors closely mirror the genotype and gene expression patterns of patient tumors [73, 74]. These "patient-derived cell lines (PDCLs)" are becoming more common in laboratory practices and are now being used in high-throughput drug screens [75]. Another area of significant development is the

in vivo models used to test drug efficacy. In the past, subcutaneous models of GBM were commonly used and tumor growth could be measured with calipers. This model does not address whether the agent can reach the tumor target, and cross the blood brain barrier. Frequently, orthotopic GBM mouse models in NOD-SCID gamma mice are being used to determine drug efficacy. Recent advances in the use of animal models for cancer research has been reported in a review written by Malaney and colleagues. The concept of "mouse avatars" and co-clinical trials is a valuable attempt to test novel or repurposed drugs on xenograft models that has the characteristics of a patient's tumor biology. This approach could revolutionize drug development and individualized therapy [76]. There are important caveats to this approach that still need to be addressed. Firstly, the mice do not have an immune system. Inflammatory cells may be a critical component to the biology of the tumor and its response to certain drugs, particularly immunotherapy. Secondly, the surrounding stroma and microenvironment is of mouse origin, not human and may interfere with drug response. Thirdly, metabolism of the drug may be different in mice and again may skew drug response.

Conclusion

GBM is one of the most problematic cancers to treat. Despite being the most common malignant primary brain tumor and the advances in molecular profiling of the disease, information is still lacking especially in the area of treatment. A one-drug fits all strategy poorly applies to GBM. Various clinical and pre-clinical trials are being conducted to investigate the effects of novel and repurposed drugs, as well as novel drug combinations. Access to a patient's genomic data through whole genome sequencing must be used to our advantage to personalize a patient's treatment. The need for pre-clinical models to validate the efficacy of these novel/repurposed drugs and drug combinations is critical. The use of patient-derived cell lines to produce orthotopic xenograft models is highly advisable. This approach could possibly give the most accurate prediction of how a drug will affect a patient's tumor and hopefully bridge the gap observed in the availability of treatments for those patients unresponsive to the current standard therapy. Additionally, a comprehensive drug-gene database would be useful to give basic researchers and clinicians a guide as to which drug-gene interaction would be highly beneficial to the patient. Personalizing treatment is a multidisciplinary approach where basic researchers, biostatisticians and clinicians play a big role in giving the right therapy to a patient.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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