Characterization of molecular pathways for targeting therapy in glioblastoma

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Abstract: Glioblastoma remains the most common malignant brain neoplasm in adults. The available therapies for treatment have only modestly extended survival. Traditional chemotherapy agents have shown only slight effectiveness in controlling this disease. The use of molecular profiling has allowed personalized medicine options to be explored for the care of these individuals. Targeted therapies have shown significant benefit in numerous other cancer types with survival being extended significantly. In glioblastoma, several promising markers have been identified including vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1). These targets have been shown to play a critical role in glioblastoma formation and proliferation. The pathways of these receptors have been elucidated in detail. This level of understanding has led to the a more robust understanding of possible mechanism of pathway modification. The targeting of these specific markers has led to the development of several selective therapies with additional therapies being evaluated. The clinical trials validating these markers have been promising but have yet to show a clear benefit in brain tumors. This identification of alternative methods to address these markers or identify additional targets may be the key to the fight against this disease. The molecular targeting of glioblastoma pathways may have significant impact on disease control and patient survival.

Keywords: Glioblastoma; targeted; glioma; epidermal growth factor receptor (EGFR); vascular endothelial growth factor (VEGF); programmed cell death protein 1 (PD-1)

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Introduction

The most common malignant primary brain neoplasm is adults is glioblastoma. The World Health Organization (WHO) classifies this as a grade four neoplasm. Glioblastoma has a median life expectancy of approximately 12 months despite current therapies. Radiation and concurrent temozolomide chemotherapy remains the standard of care initial therapy after surgery (1). This treatment regimen has had a significant impact on progression-free survival (PFS) and overall survival. Novo-TTF, a non-pharmaceutical intervention, has also shown improved outcomes in glioblastoma treatment (2). Bevacizumab, although received accelerated approval for the treatment of recurrent glioblastoma, efficacy continues to remain unclear. Novel and more precise advances have provided new optimism.

Chemotherapy has traditionally focused non-specific mechanisms to prevent cell growth, this focused heavily on the inhibition of DNA replication. The mechanisms tumors can replicate and survive has been robustly explored. The understanding of molecular basis for cancer proliferation has changed our treatment strategy to specific molecularly targeted therapies. Potentially, tumors with aberrant signaling in one pathway are more likely to respond to

agents targeting that pathway. Agents that target a different pathway would like less effect in treating this tumor. This is described personalized medicine in that treatment is targeted to molecular profiles unique to the cancer. Intense research into clinical trials that are no aimed at determining these molecularly targeted agents which may benefit patients with glioblastoma (3).

Standard therapy for glioblastoma

Surgical resection is the first step in the diagnosis and treatment of glioblastoma. Subsequently, standard protocol includes radiation therapy with concurrent temozolomide. Patients are subsequently treated with adjuvant temozolomide therapy for a minimum of 6 cycles. This standard therapy has shown an overall survival of 14.6 months with a median PFS of 6.9 months and a 25.6% 2-year survival rate (1). These lackluster findings demonstrate that glioblastoma continues to have a grim prognosis. The treatment for recurrent glioblastoma has not standard of care option as no clear efficacious therapy has emerged. Traditional chemotherapies have long been evaluated without a significant impact on survival. The most frequently used therapies include, carmustine, carboplatin, irinotecan, BCNU wafers, or repeat surgical intervention. These recurrent treatment options have minimal survival impact. However, without any clear alternatives they remain the most widely used options to date.

Genetic variations in glioblastoma

Many mutations have been shown to have a role in the development of glioblastoma. The Tumor Cancer Genome Atlas (TCGA) has shown three main pathways activated in most glioblastoma tumors. These are the epidermal growth factor receptor (EGFR), retinoblastoma (RB), and p53 pathways (4). Numerous clinical trials have attempted to address the components of these pathways but none have shown any clinical efficacy (3). The development of an effective targeted therapy has been challenging given the heterogeneity in glioblastoma. Bevacizumab remains the only targetable therapy which has shown significant response and possible clinical efficacy.

EGFR is an upstream receptor that is activated by the binding of epidermal growth factor. In glioblastoma, there is often a ligand-independent mutation of the receptor, called EGFRvIII, which is constitutively active (5-14). The activation of this receptor results in recruitment of phosphoinositide 3-kinase (PI3K) to the cell membrane. Phosphatidylinositol (PI)-4,5-bisphosphate is phosphorylated to PI-3-phosphate (PIP3) by PI3K. PIP3 subsequently activates downstream molecules such as protein kinase B (AKT) and mTOR (15). Cell proliferation and inhibition of apoptosis is the result of this signaling cascade. Phosphate and tensin homology (PTEN) serves as a checkpoint in this system by inhibiting PIP3 signaling. It also has homology to the catalytic region of protein tyrosine phosphates which are important to the function of PIP3. The gene for PTEN is located at 10q23.3. In 15–40% of glioblastomas there is mutation of this gene which supports the assumption that deregulation of this pathway is common is glioblastoma (12,16,17). EGFR signal transduction also stimulates Ras proteins. Ras proteins are membrane associated GTPases which require post-translational addition of a farnesyl group to the C-terminus. This is accomplished by farnesyl transferase (18). Ras proteins, once activated, stimulate cellular proliferation, survival and angiogenesis (19). The Ras protein and farnesyl transferase are potential targets for inhibiting the EGFR pathway.

The p53 pathway is mutated in 87% of glioblastomas (4). The transformation of lower grade gliomas to glioblastoma is proposed to involve mutation in the p52 pathway. TP53 stimulates apoptosis or senescence as a response to DNA damage. The *TP53* gene is located at chromosome 17p13.1 and causes loss of regulatory control if mutation or homozygous deletion occurs (20). Murine double minute 2 (MDM2), which inhibits cells from entering apoptosis, binds TP53 blockings its ability to activate transcription of promoter sequences (21). This in turn essentially may cause glioblastoma cells to gain immortality by amplification of MDM2. The *AFR* gene product lies upstream of MDM2 which can bind to it. ARF binding of MDM2 inhibits the function of p53 (22-24). Homozygous deletion or promoter methylation of this gene product has been found frequently in glioblastoma leading to loss of expression. Promotor methylation is seen both primary glioblastoma and glioblastoma which as transformed form a lower grade glioma (secondary glioblastoma) (25). Disruption of any of these MDM2, TP53, or ARF products can cause cells to lose normal function because there is a strong autoregulatory feedback between these genes.

The RB protein is required for progression through the cell cycle. Mutations in the RB pathway have been found in 78% of glioblastomas (4). The RB protein is required for the cell to progress form G1 phase to S phase. RB inhibits E2F transcription factor which activates genes

involved with transition between phases (26). The RB protein is phosphorylated by the CDK4/CCND2/CDK6 complexes which inhibits it activity. CDK4/CCND2/CDK6 amplification is seen in 1–18% of glioblastomas. Cell replication increase as a result of this amplification. P16 and CDKN2B act to inhibit these complexes and lie upstream of these complexes. Alteration in the expression of any of these genes, RB, or CDK4 complexes can lead to uncontrolled cell division.

Glioblastoma therapeutics are now focused on the development of targets for many of these genes, proteins, pathways, and complexes. It is known that certain subgroups of glioblastoma patients respond divergently to targeted treatment options. Glioblastoma cells have variable molecular genetic patterns that confer aberrant abilities to replicate cells. While multiple pathways may be present in a glioblastoma often times there appears to be dominant process. If a targeted agent to affect this dominant pathway it would have a significant impact of tumor growth and proliferation. This effect would be limited to the subgroup that showed this pathway and not effective in subgroup that was driven by an alternative dominant pathway. Thus, the need for personalized medicine and the use of molecularly targeted interventions.

Vascular endothelial growth factor (VEGF) molecular targeted therapy

Glioblastoma is known to shown increased VEGF production. VEGF stimulates vascular proliferation in the area surrounding glioblastoma cells. The result is increased nutrient supply, oxygen delivery, and blood flow. Glioblastoma growth and proliferation is enhanced by these factors. It has been shown that high levels of VEGF in gliomas are associated with a worse prognosis (27).

Bevacizumab was the first targeted therapy approved for the treatment of glioblastoma. Bevacizumab is a humanized monoclonal antibody against VEGF. Bevacizumab inhibits the VEGF signaling pathway preventing increased vascular supply to glioblastoma cells. The Avaglio study was a phase 3 randomized controlled study investigating the use of this agent in newly diagnosed glioblastoma patients. In this study 458 patients received bevacizumab while 463 patients received placebo. The study revealed a median PFS of 10.6 months in the bevacizumab group versus 6.2 months in the placebo group [stratified hazard ratio for progression or death, 0.64; 95% confidence interval (CI), 0.55 to 0.74; P<0.001] (28). However, overall survival did not differ significantly between the two groups (stratified hazard ratio for death, 0.88; 95% CI, 0.76 to 1.02; P=0.10) (28). Overall survival at one year with bevacizumab was 72.4% and placebo was 66.3% (P=0.049) Overall survival was respectively 33.9% and 30.1% at 2 years (P=0.24) (28). The bevacizumab group maintained health-related quality of life and performance status longer. They also had a lower glucocorticoid requirement. There was in increased incidence of grade 3 or higher adverse events in the bevacizumab group versus the placebo group (66.8% versus 51.3%) (28). This large study shows the addition of bevacizumab to standard of care with radiotherapy and temozolomide did not improve survival in patients with glioblastoma. The patients had a significantly longer PFS and maintain a higher performance status and quality of life in the bevacizumab arm. They also had a higher incidence of adverse events.

Despite these results the targeting of VEGF continues to be a pathway of intense optimism. The PFS benefits seen in the phase 3 trial and reports in numerous other publications keep bevacizumab a frequently used option in recurrent glioblastoma. The use of combination therapies with bevacizumab and traditional chemotherapy options is also being explored. New VEGF targeting agents have also started to enter clinical trials.

Programmed cell death protein 1 (PD-1)/ programmed death-ligand 1 (PD-L1) checkpoint blockage

The PD-1/PD-L1 pathway suppresses the function of T cells in removing tumor cells (29-31). Several types of solid tumors have shown upregulation of PD-L1. Tumors that shows a high expression levels of PD-L1 often have better outcomes with PD-1/PD-L1 checkpoint blockades (32-35). These findings lead to the evaluation of PD-L1 expression and the clinical efficacy of checkpoint blockades in glioblastoma patients.

Newly diagnosed glioblastoma has increased PD-L1 expression in 88% of specimens. Recurrent glioblastoma specimens 72.2% of probability of PD-L1 overexpression (36). One study showed that 61% glioblastoma patients had PD-L1 overexpression (37). The principal was tested in the Checkmate 143 clinical trial. The trial investigated the use of nivolumab, PD-L1 blocking agent with or without ipilimumab, a cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) blocking agent. The phase 1 cohort of the study evaluated the clinical effects and tolerability in addition

to the safety of nivolumab with or without ipilimumab in patients with recurrent glioblastoma. Forty patients in total where evaluated. Ten patients were randomized to receive nivolumab as a single agent, ten patients received low-dose nivolumab and low-dose ipilimumab, and the other twenty patients received high-dose nivolumab and high-dose ipilimumab (38). The subgroup receiving nivolumab alone tolerated the treatment better than other two subgroups (38). Fatigue and diarrhea were the most common treatment-related adverse events. The study was also found that the dose of the higher dose ipilimumab was correlated with poorer patient tolerance (38). The poor tolerance may be explained because ipilimumab has an earlier impact on T cell activation. This activation results in an extensive impact in the immune network (38).

Checkmate 143 proceed onto a phase three clinical trial. The study compared nivolumab with bevacizumab therapy in recurrent glioblastoma patients who had failed standard of care upfront therapy with radiation and temozolomide (39). This study revealed nivolumab alone did not have an impact overall survival. The median PFS was 1.5 months for nivolumab versus 3.5 months for bevacizumab (39). The median overall survival was 9.8 months for nivolumab versus 10.0 months for bevacizumab (39). The objective response rate (ORR) has assessed by magnetic resonance imaging was 8 versus 23 months in the nivolumab and bevacizumab arms respectively (39). Lymphopenia caused by radiotherapy was hypothesized as a plausible etiology of the nivolumab monotherapy ineffectiveness. Radiotherapy has been shown to have an effect on circulating lymphocytes decreasing they function (40). Nivolumab monotherapy works by competitively binding PD-1 receptors so a decrease functional lymphocyte count and reduce potential targets. An addition hypothesis for the subdued response of nivolumab is that effector T cells are anergic to specific antigens. The lymphocytes in gliomas specimens typically possess CD95, PD-1, PD-L1, CTLA-4, lymphocyte-activation gene 3 (LAG3), and T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) antigens (41). Tumor infiltrating lymphocytes (TILs) express immunoinhibitory molecules, including CTLA-4 and PD-1, or co-express PD-1 and TIM-3. These targets may be exhausted on T cell population in tumors (42,43). The T cell anergy in glioblastoma by we too great for PD-1 checkpoint blockage to overcome on its own. Further studies with combination therapy remain ongoing.

PD-1 and PD-L1 are one of the first targeted therapies to utilize the immune system in the battle against cancer. This approach has not only advanced targeted options despite the negative results in glioblastoma it has also open the door to other possible immunotherapies. The immune pathway offers a multitude of potential mechanism of action that remain to be explored.

EGFR

Genetic alterations in receptor tyrosine kinase (RTK) signaling pathways are present in the majority of glioblastomas (44). Among the most relevant pathways is EGFR activation (45). EGFR amplification, mutation, rearrangement, altered splicing or genetic alteration is seen in 57% of glioblastoma (44-48). The majority of these cases had regional DNA amplification. This led to a wide range of mutation allelic frequencies. Comparing the allelic frequencies of point mutations in DNA- and RNA-seq data revealed a high degree of concordance between the type and prevalence of mutations at the DNA level and the composition of expressed mRNA transcripts. A complete picture of aberrant exon junctions and a semi-quantitative assessment of their expression levels was also provided by RNA-seq (44).

Transcript allelic fraction (TAF) was calculated as the ratio of each aberrant exon junction to the sum of aberrant and wild-type junctions at the 3' junction end. In 11% of tumors, the aberrant exon 1–8 junction characteristic of EGFRvIII was highly expressed. At least 19% of tumors showed low level expression (≥1%). Digital mRNA assay was able to come to the same conclusion. EGFRvIII expression in glioblastoma is associated with an aggressive tumor phenotype through paracrine mechanism (49).

A variety of other recurrent noncanonical EGFR transcript forms were detected in the RNA-seq data. Three different C-terminal rearrangements targeting the cytoplasmic domain of the EGFR were detected at ≥10% TAF in 3.7% of cases and at ≥1% TAF in another 9%. Whole genome sequencing (WGS) data confirmed the presence of C-terminal deletions. These variants have previously been associated with glioma formation in experimental rodent systems *in vivo* (50). The complete loss of the C terminus may yield aberrant terminal junctions not able to be mapped by transcriptome sequencing. Under-expression of C terminus exons 27–29 (<3 SD) were apparent in another 7.3% of cases without detectable aberrant junctions.

Two relatively uncharacterized recurrent EGFR variants, namely deletions of exons 12–13 (Δ12–13) in 28.7% and

exons 14–15 (Δ14–15) in 3% have also been identified. EGFR Δ12–13 has been previously identified by RT-PCR analysis of glioma (51). Both Δ12–13 and Δ14–15 appear to be expressed in minor allelic fractions (<10%). This finding raises the question of whether they result from genomic deletion or splicing aberration. WGS analysis of aberrant junctions did not shows a concordant DNA deletion in tumors expressing Δ12–13 mRNA. This data suggests concordant breakpoint as a minor component of a highly rearranged locus. By comparison, EGFRvIII-expressing tumors had concordant deletion spanning exons 2–7 in WGS data (51).

EGFR amplification is frequently associated with a deletion mutation affecting exons 2–7, referred to as EGFRvIII or delta-EGFR. Twenty to thirty percent of all glioblastomas show EGFRvIII expression (44,52,53). The potential immunogenicity of the EGFRvIII mutation leads to the production of peptide vaccine, rindopepimut, containing the specific novel amino acid sequence created by the EGFRvIII deletion mutation conjugated to keyhole limpet haemocyanin. Rindopepimut was evaluated in two phase 2 trials, ACTIVATE (54) and ACT II (55), as well as a larger phase 2 trial ACT III (56). Collectively, about 100 patients newly diagnosed EGFRvIII expressing glioblastoma treated with a gross total resection and standard of care chemo-radiation were given single agent rindopepimut (ACTIVATE) or rindopepimut plus adjuvant temozolomide (ACT II and ACT III). These trials showed a 15-month PFS and a 24-month overall survival from time of diagnosis (52,57). These results compared favorably with contemporary patient cohorts who received standard treatment. The selection of patients with minimal residual disease in all these trials after completion of chemoradiation assumed that it would minimize the tumor-associated immunosuppression typical of glioblastoma.

The ACT IV study investigated newly diagnosed glioblastoma patient treated with rindopepimut versus a placebo control. The study failed to show an overall survival benefit for either the minimal residual disease or intent to treat populations. Median overall survival in the rindopepimut group was 20.1 versus 20.0 months in the control group (57). In the minimal residual disease population and 17.4 versus 17.4 months, respectively (57). In an exploratory analysis of the stable residual disease population median overall survival was similar between the rindopepimut treatment groups 14.8 versus 14.1 months in the control group (57). These studies investigating the use of an EGFRvIII antagonist did not show a benefit for glioblastoma patients. Direct EGFRvIII antagonism not mediated by an antibody and EGFR antagonist remain to be explored.

Conclusions

The identification of molecular pathways to cancer formation and treatment has changed the landscape of oncology. The molecular targets identified in glioblastoma have expanded our understanding of the disease process. The VEGF receptor, EGFR, PD-1/PD-L1 pathway have been established is driving mechanisms of glioblastoma formation and growth. Each of these pathways has led to the discovery of multiple potential targets. The targeted therapies for these pathways are aggressively being developed and continue to push into clinical trials. First generation drugs have proceeded to large scale clinical trials. Unfortunately, a clear treatment efficacy remains elusive at this time. The trials each show significant promise but failed to meet set efficacy endpoints. However, the elucidation of these targets has opened the path to the development of new options. There are ongoing trials investigating neurotrophin signaling, BRAF, NTRK, and MET targets currently. These may be used in combination to block more than one pathway in a single tumor. The use of targeted therapies has already changed many oncology treatment paradigms. The landmark breakthrough in glioblastoma therapy may be in the near future.

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