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A Glioblastoma Genomics Primer for Clinicians

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Abstract

Abstract: New discoveries in Glioblastoma (GBM) biology have been made using genomics data. Genomic markers are routinely integrated into clinical neurosurgical practice. In this manuscript, we review the fundamentals of genomics such as the differences between first, second, and third generation sequencing technology. We also review the impact of single cell genomics in understanding the complex heterogenous GBM microenvironment. Finally, we will discuss advances in epigenetics that have lent insights into treatment resistance. The integration of genomics into neuro-oncology clinical practice is routine and will continue to expand with the expansion of precision of medicine. We provide a primer for clinicians.

Keywords: epigenetics; glioblastoma; genomics; microenvironment; Single-Cell Analysis; tumor microenvironment

1. Introduction

Glioblastoma Multiforme (GBM) remains the most common malignant primary brain tumor among adults and still portends a median survival of 15 months following standard therapy known as the Stupp protocol (i.e. surgery followed by radiation and temozolomide treatment). Despite over a decade of advances in cancer biology and corresponding clinical trials, little changes have been made in standard therapy for GBM since 2005[1,2]. While glioblastoma remains one of the most malignant tumors of the body, likely due to a wide range of inter- and intra-tumor genetic heterogeneity contributing to treatment resistance, advances in technology have allowed for better understanding of the disease and classification.

In the early 2000's, bioinformatics began developing as a field that uses computational methods to analyze data from genomic sequencing, proteomics, and systems biology[3]. Many researchers began to use bioinformatic techniques to understand the complexities of cancer biology including GBM. This use of integrated genotypic and phenotypic characteristics was one of the major updates to from the 2007 WHO CNS classifications to the 2016, updated 4th edition[4]. With this update, *IDH* status became key in the classification of GBM. *IDH*-wildtype represents the vast majority of cases clinically defined as primary/de novo glioblastoma, often occurring in patients later in life. *IDH*-mutant corresponds with "secondary glioblastoma", patients with a history of prior low grade diffuse glioma, which arises earlier in life[5].

Advances in genetic sequencing have also provided new insights into understanding GBM biology and the tumor microenvironment. These insights will hopefully lead to improvement in treatment, but there remains a chasm between clinicians and the practical utilization of the ever-growing field of genomics. In this review, we discuss the currently used genetic predictive markers in GBM and provide a primer in understanding genomics for clinicians. As the cost of genome sequencing continues to drop, the amount of data created is quickly becoming 'big data', on the same level as astronomy and other large-scale physics data [6]. A basic understanding of genomics will be necessary to navigate the future of neuro-oncology practice.

2. Current Clinically Used Molecular Markers

2.1. IDH Mutation Status

Molecular markers have become routine to use clinically for the evaluation and treatment of GBM. Mutations in the Isocitrate Dehydrogenase 1 or 2 (*IDH*1/2) gene have

become an important molecular biomarker used clinically. In 2016, the world health organization's summary on tumors of the central nervous system incorporated *IDH* mutational status into the classification of GBM [7]. Somatic mutations in these genes were frequently identified in patients with secondary glioblastoma (i.e. tumors that evolved from low grade gliomas)[5]. *IDH*1/2 mutations were found to be present in over 70% of low grade gliomas and secondary glioblastomas and do portend a better prognosis [8,9]. Furthermore, *IDH* mutant gliomas undergo chromosomal scattering to a much larger extent and have distinct oncogenic pathways in comparison to *IDH* wildtype (wt) tumors [10].

Work by Lu et. al. was instrumental in elucidating the mechanisms of *IDH* mutations[11]. The *IDH* genes encode the enzymes needed for decarboxylation of isocitrate to α-ketoglutarate. These mutations lead to the production of a oncometabolite, 2-hydroxyglutarate (2-HG), that can modify cellular processes, such as, inhibition of histone demethylation that blocks the differentiation of non-transformed cells[11,12]. Levels of 2-HG correlate with increased mitotic activity, axonal disruption and vascular neoplasia [13].

Clinically, *IDH* mutational status confers a better prognosis and has become incorporated into predictive prognostic nomograms for patients with primary GBM [14]. Studies of *IDH*-wt tumors demonstrate that *IDH*1 upregulation supports aggressive growth and treatment resistance in GBM [15]. *IDH*-wt patients with a mutation in TElomerase Reverse Transcriptase gene promoter (TERTp) have a poor prognosis [16]. Among *IDH*-wt patients, extent of surgical resection and MGMT promoter methylation status can still be used to predict overall and progression free survival [17].

2.2. MGMT Promoter Methylation Status

Determination of the promoter methylation status of O(6)-Methylguanine-DNA Methyltransferase (MGMT) is another main prognostic indicator used by clinicians. Temozolomide, a DNA akylating agent, remains the primary chemotherapy in GBM and is used as part of standard treatment. Resistance to this chemotherapeutic can be mediated by the MGMT DNA repair enzyme and methylation of MGMT leads to silencing of this gene. MGMT methylated patients are therefore more responsive to the chemotherapy [18].

MGMT promoter methylation status has correlated with survival and treatment response in GBM patients. In the Nordic randomized clinical trial, which enrolled elderly patients with GBM, MGMT

methylation status was a predictive marker to determine responsiveness to temozolomide treatment [19,20]. Based on these results, some clinicians opt to forego temozolomide, however, there remains wide variability in practice paradigms globally [21]. The evidence on this topic remains weak, therefore, use vs withholding of temozolomide in the elderly based on MGMT status alone is not recommended. Further consensus is needed to determine when temozolomide treatment is futile in the elderly popultion.

Further argument that MGMT methylation status should not be used as a sole guide for treatment and prognosis lies in the understanding of how the methylation status is assessed. Multiple methods exist for detection of MGMT methylation and cutoff values are also different among institutions. Immunohistochemistry is one method to assess methylation status, but there is concern for interobserver variability and how pathological processing can affect antigen specific binding [22]. Methylation specific polymerase chain reaction (PCR) is the most common detection method, but optimal cutoff values remain controversial, and there is no standard value of methylation that correlates to a difference in clinical response. Furthermore, multiple trials have used different definitions for determining methylation status [23]. Thus, it is important for

clinicians to be aware of these limitations when deciding on the utility of temozolomide for certain patient populations. MGMT methylation status is not the only predictive biomarker and a comprehensive assessment is still needed.

2.3 Further Genetic Molecular Markers

Thus far, *IDH* mutant status and MGMT methylation and are the main molecular markers that are used clinically to predict prognosis. However, clinicians should be aware of new genetic markers and subsets of GBM given their relevance to many ongoing clinical trials and their potential for further prediction of outcome. Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase receptor that is known to affect GBM development and progression. Amplification of the EGFR gene is present in up to 45% of GBMs. The variant III mutation of the epidermal growth factor receptor (EGFRvIII) is a neoantigen and can be used as a therapeutic target [24,25]. EGFR expression has also been found to be a prognostic biomarker in some cohorts. A subgroup of patients with both unmethylated MGMT promoter status and high EGFR expression have the shortest survival in comparison to patients with other variations of these two markers [26].

Mutations affecting the TERT promoter are also very common in GBM [27]. Patients

with a mutation in the TERT promoter had a shorter overall survival compared to TERT promoter-wt (13.8 months vs 18.4 months)[28]. In this cohort, when EGFR amplification or TERT promoter mutation was present, prognosis was poor (less than 1.5 years). For patients without these molecular markers, prognosis was much better especially for the *IDH* mutant patients (survival of more than 3 years) [28]. These studies demonstrate that the best prognostication will be by combining the implications of multiple markers [29].

3. Implications of GBM Genomics

3.1 Next Generation Sequencing in GBM

All of these aforementioned genetic changes and markers in GBM have primarily been due to the advances in technology that have decreased cost and improved the speed of genetic sequencing techniques. With this new speed and relative affordability, analysis of large enough numbers of tissue samples to give statistically significant results is now possible.

After the sequencing of the first proteins in the 1950s, critical refinement of cumbersome techniques led to the development of "first- generation" sequencing technology (figure 1) that allowed the first sequencing of the human genome in the 1970s[30]. This "first- generation" sequencing continued to be automated and improved. By 2005 (the same year as the development of the Stupp protocol for GBM treatment) a more high throughput system gave rise to "second-generation" also referred to as "next generation" sequencing (figure 1). This sequencing method is still the most commonly used technique in labs due to its availability, reliability, and high output. Developments in nanotechnology has ushered in third generation sequencing, which uses single molecule sequencing technology, without the need of DNA synthesis. While challenges still exist in implementing widespread use of third gen sequencing, it provides the hope of minimal pre-processing of samples and potential obtain direct detection of epigenetic markers [31,32] (Figure 1).
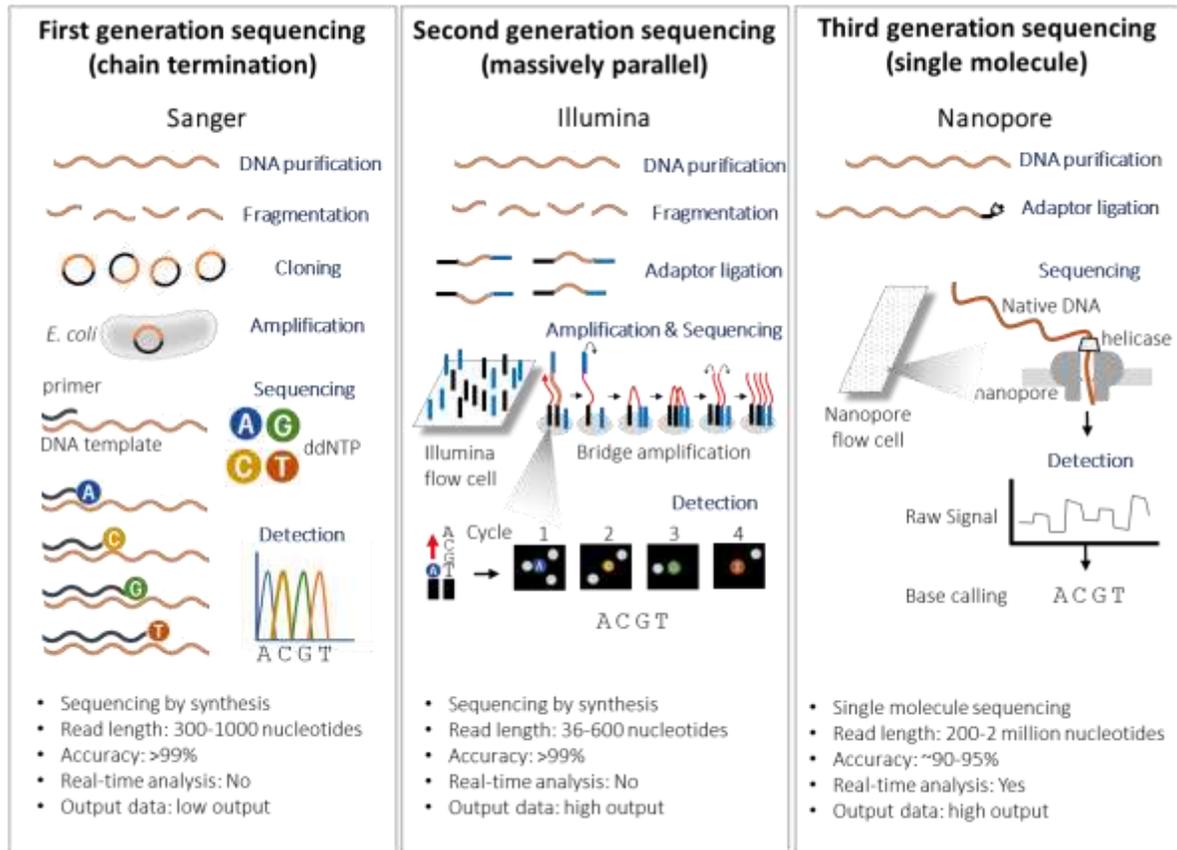
Figure 1. Schematic Representation of first, second and third generation sequencing.

With this boom in available genetic data for analysis, GBM was the first cancer to be studied by the cancer genome atlas research network with the analysis of 206 samples in 2008. Genetic alterations identified in three main signaling pathways (RTK/RAS/PI-3K, p53, and RB) allowed for elucidation of gliomagenesis [33,34]. Distinct pathways discovered are also involved in the development of supratentorial versus infratentorial GBM, implying that the pathogenesis of cerebellar GBM requires unique gliomagenesis pathways [35]. Through collaborative efforts, many genomic sequences from GBM samples are now publicly available and can be analyzed using bioinformatic strategies. This collaborative analysis identified differential gene expression levels that were associated with GBM, in comparison to normal tissue samples, that were found to significantly affect prognosis [36]. Further analyses of gene expression profiles from 1280 GBM patient samples found druggable genes (such as RRM2, MAPK9, and XIAP) that are related

to carcinogenesis, giving new insight into the development of novel therapies[37].

Advances in sequencing methodologies allowing sequencing on formalin fixed paraffin embedded (FFPE) tissue have made tumor genetics clinically important for all clinicians. This allows genetic sequencing to be part of the routine pathological analysis of tissue samples following surgical resection, as well as retrospective analysis on previously resected tumors. For clinicians, FFPE specimens can be sent to commercial vendors for genomic profiling, with information available in as little as five days [38,39]. Typically, these vendors conduct an analysis on a panel of genes rather than the whole exome and summarize the findings for clinicians in order to match candidate patients to clinical trials. For some clinicians, sending genomic sequencing following surgical resection of de novo and recurrent tumors has become routine and is done prospectively [40,41]. This allows for the identification of patients who may qualify for targeted therapy and/or clinical trials.

IDH mutational status is required for diagnosis and therefore is integrated into the pathology report of patients. At our institution, all glioblastoma patients also get tested for MGMT methylation and EGFR amplification . Since these biomarkers are integrated into the pathology workflow, they are often billed to and paid by insurance. Further genetic sequencing can be carried out by sending clinical samples to third-party companies. These sequencing data are typically filtered to provide a clinical report matching the patient to potential clinical trials. Medicare often covers these expenses for patients who qualify but private insurers may or may not pay for the extra sequencing costs. However many companies offer financial assistance.

3.2 Single Cell Sequencing and Tumor Immune Microenvironment

In genetic sequencing of spatially different regions of a GBM tumor, it was discovered that multiple subtypes were present within the same tumor [42]. GBM is now known to be a highly heterogenous tumor, with multiple cell types, and a microenvironment with different regional niches of varying functions [43]. Thus, a main hurdle in the understanding of GBM is this heterogeneity, as sequencing from a portion of the tumor bulk can lead to a biased understanding of the genetic cell lineages from different cell populations especially in GBM [44]. In order to study genomics on a cellular level, single cell transcriptome

analysis became possible in 2009 [45,46]. Now it is possible to understand the gene expression profiles from individual cells rather than a pool of cells. Single cell sequencing is therefore being used to understand subgroups within heterogenous tumors, making its application in GBM vital.

Single cell sequencing has already provided vital information to the pathophysiology of Glioblastoma. In one study, GBM samples demonstrated diverse ontogenic pathways and phenotype variability on the cellular level [47]. Cells from the subventricular zone were also discovered that possess driver mutations, which guide the migration of these cells to other brain regions to develop into GBM [48]. Infiltrating glial cancer cells at the migrating front of a GBM tumor also share gene signatures that give insight into mechanisms of glioma cell infiltration, a key pathological hallmark[49]. Transcriptionally distinct signatures were also present in myeloid populations residing in the tumor core and surrounding peritumoral space, highlighting the role of the tumor immune microenvironment[49].

Thus, the glioma tumor microenvironment is diverse and composed of glioma cancer cells as well as stromal cells, multiple infiltrating inflammatory and immune cells, and stem cells [50,51]. Of these, Tumor/Glioma Associated Macrophages/Microglia (GAMs) have been shown to account for up to 50% of the tumor cell mass[52–54]. The role of GAMs in the progression of GBM remains to be fully elucidated, but it is known that GAMs aid in glioma cancer cell proliferation, neo-vascularization, and invasion [55]. GAMs and Myeloid Derived Suppressor Cells (MDSCs) also play a large role in directing immunosuppressive responses, which hinder efforts to employ immunotherapy as a treatment for GBM [56,57]. Transcriptome analysis of human GAMs found expression signatures of upregulated genes necessary for cell migration, adhesion, and extracellular matrix organization [58]. With this myeloid upregulation, studies have also demonstrated that GBM has a lymphocyte depleted immune landscape[59].

Clinically, these insights into the GBM microenvironment provided by collaborative efforts between neurosurgeons and scientists are essential to integrate clinical trials and new therapies, especially immunotherapy, as the tumor immune microenvironment is one of the main hindrances to application of immunotherapy, such as CAR-T cell or NK therapy, in solid CNS tumors.

3.3 Glioblastoma Epigenetics

Epigenetics refers to the DNA modifications that affect gene activity without altering the DNA sequence itself. Two very popular and well-studied mechanisms for epigenetics are DNA methylation and post-translational modification of proteins (often histone modifications are studied, because these can have broad regulatory effects on gene expression levels). Epigenetic changes are integral for tumor progression and in mediating chemotherapy resistance [60]. The most common example of this in GBM is related to the MGMT promoter methylation status which determines sensitivity or resistance to DNA alkylating agents as previously discussed in this review.

Recently, genome scale methylation maps of over one hundred GBM samples were constructed and linked to histopathological and radiographic data[61]. DNA methylation was linked to tumor immune cell infiltration and the microenvironment, and is largely preserved between primary and recurrent tumors [61]. Further characterization of epigenetic changes in GBM have demonstrated that tumors with the CpG Island Methylator Phenotype (CIMP) have epigenomic aberrations that are related to pathogenicity[62]. How this phenotype is developed is not well understood, but *IDH* mutations are associated with widespread changes in the methylome which leads to activation of genetic processes[62].

In cancer stem cells (also known as cancer progenitor cells), it has been demonstrated that epigenetic changes are critical in mediating differentiation and immortality of tumor cells [63]. Key studies in GBM have shown cancer stem cells are resistant to radiation and chemotherapy and give rise to the cells that facilitate recurrence[64,65]. These epigenetic signatures found in cancer stem cells, which can even be unique among GBM subtypes, are related to important cellular pathways, such as DNA damage responses[66,67].

Currently, epigenetic studies remain laborious and lack the feasibility to be carried out routinely, but one of the potential advantages to come of third generation sequencing, as previously discussed, is the possibility to obtain these methylation patterns routinely. Increased feasibility of epigenetic studies are important as epigenetic modulation may be a key to targeting treatment resistant glioma cell populations.

4. Conclusions

Genomics has been revolutionary in transforming the understanding of Glioblastoma biology. Bioinformatics and collaborative efforts between surgeons and scientists has been critical in leveraging the data and allowed for both the retrospective and prospective application of next generation sequencing. Molecular markers and mutational status has revolutionized the way we educate, plan, and treat patients with GBM. Advances, such as single cell sequencing, have furthered our understanding of the heterogeneity within GBM, and its complicated microenvironment. Studies in epigenetics and cancer stem cells have highlighted the importance these small molecular changes can make in determining tumor cell resilience and recurrence. These efforts are driving personalized treatment and prediction of survival and can be further be enhanced by the use of novel personalized treatment approaches. The use of patient derived organoids to assay for therapeutic sensitivity [68], and sequencing of circulating serum and cerebrospinal fluid biomarkers in GBM patients also provides the possibility of non-invasive disease course monitoring [69,70] (Figure 2 outlines the likely neuro-oncology workflow of the future for patients with GBM).

GBM remains a deadly tumor with minimal treatment options available. Higher order bioinformatic genetic processing of patient samples and integration with clinical parameters have allowed for novel insights into gliomagenesis. Beyond characterization and survival prediction, the next steps are to provide personalized therapies for patients.
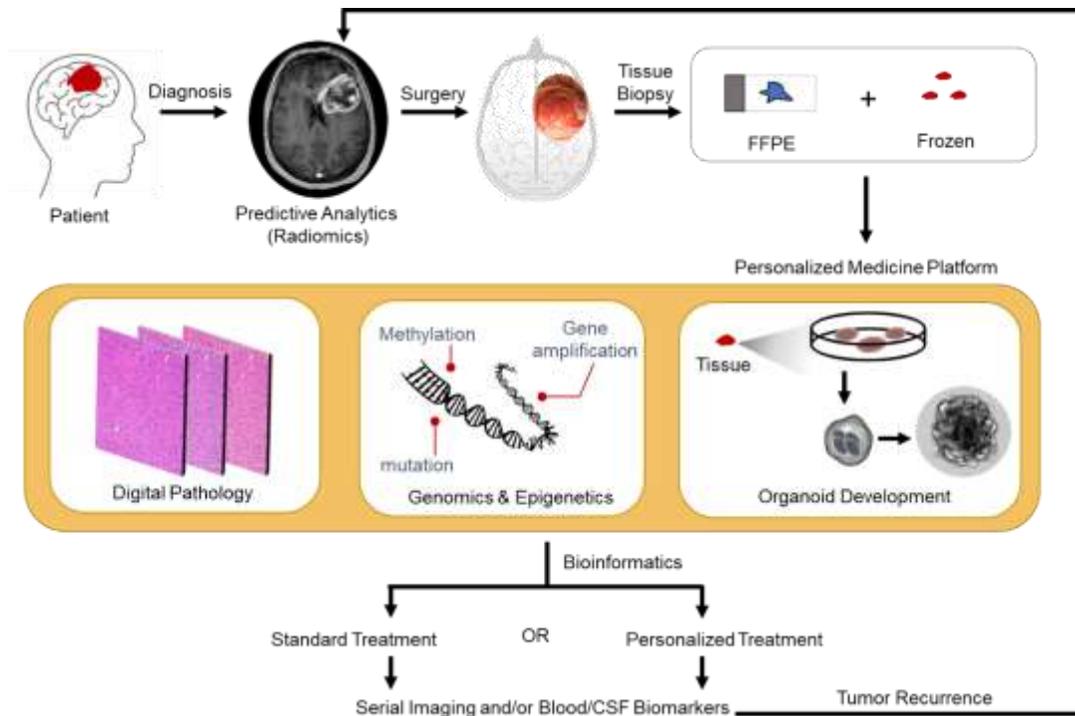
Figure 2. Schematic of the Future of Neuro-Oncology Practice following diagnosis of GBM. Patients will likely have preoperative screening with radiomics and from tumor tissue have an array of assays designed for personalized medicine. This process will be iterative as recurrence remains inevitable.

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