Chapter 4

Genetic Profiling: Searching for Novel Genetic Aberrations in Glioblastoma

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1. Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumors and remains one of the deadliest of human cancers [1]. The incidence of this cancer is fairly low, with 2-3 cases per 100,000 people in Europe and North America. GBM is slightly more common in whites than in blacks, Latinos, and Asians, with a slight male predominance - M:F ratio of 3:2 [2]. The overall prognosis for GBM has changed little in the past two decades, despite major improvements in neuroimaging, neurosurgery, radiation treatment techniques, adjuvant chemotherapy, and supportive care. Without treatment, the median survival is approximately 3 months [3]. The current standard of care involves maximal surgical resection followed by concurrent radiation and chemotherapy with the DNA alkylating agent temozolomide [4]. Despite this aggressive regimen, the median survival remains approximately 14 months. Thus, meaningful strategies for therapeutic intervention are desperately needed.

The most reliable evidence suggests that glioblastomas originate from cells that give rise to glial cells [5, 6]. The World Health Organization (WHO) classifies these glial-derived tumors into four major categories, namely WHO grade I-IV. The higher grade signifies patho-histologic features of increased malignancy. WHO grade IV glioma is synonymous with glioblastoma [7].

Rigorous scientific investigations over the past three decades indicate that glioblastomas, similar to other cancers, are the stem from collection of genetic alterations. These alterations can present in a variety of forms, including epigenetic alterations, point mutations, translocations, amplification or deletions – resulting in gene modifications. The genetic alteration results in either activation or inactivation of specific gene functions that may contribute to the process of carcinogenesis [8]. Those genes, that when activated, contribute to the development of cancer are often termed proto-oncogenes. The mutated forms of these

genes are referred to as oncogenes. Conversely, genes that when inactivated contribute to carcinogenesis are generally termed tumor suppressor genes. Although it is well established that central nervous system (CNS) carcinogenesis requires multiple deregulations of the normal cellular circuitry, the exact number and nature of genetic alterations and deregulated signaling pathways required for tumorigenesis remains subject of ongoing scientific investigations [9].

1.1. Cancer genomic era

The current decade will likely be remembered, in the history of cancer research, as the decade of cancer genomics. The marriage of technology and annotated specimen collection has culminated to provide us with a glimpse of the complex genomic landscape that underlies cancer pathogenesis. Remarkably, these efforts have demonstrated true collaborative spirits between clinicians and basic science researchers with common goals of furthering translational science.

The Cancer Genome Atlas (TCGA) constitutes the largest of the genomic efforts. It is a comprehensive and coordinated effort to accelerate our understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing. This is accomplished via cataloguing the genetic and epigenetic changes in the cancer genome, with goals of identifying those responsible for carcinogenesis. The project represents a joint effort of the National Human Genome Research Institute (NHGRI), National Cancer Institute (NCI), the U.S. Department of Health and Human Services, and collects of tumor specimen from major cancer centers spanning across the continental USA. The project aims to provide the genomic profile of 500 specimens of various cancer types using state-of-the-art platforms for sequencing, microRNA, mRNA, single- nucleotide polymorphisms (SNPs), and methylation profiling. TCGA started as a pilot project in 2006 with focus on glioblastoma as the first cancer type for study. With the success of the pilot project, TCGA has committed to expand its efforts to aggressively pursue 20 or more additional cancers. While acknowledging the importance of the TCGA in cancer research, one cannot neglect the value of the pioneering genomic efforts that, in many ways, laid the groundwork for the TCGA [10]. The knowledge to sequence the entire genomes of human tumors including glioblastoma, helps formulating new concepts and principles in tumor cell biology, and enables potential exploitation of these major advances for personalized disease management in oncology.

With advances in genomic profiling and sequencing technology, we are beginning to understand the landscape of the genetic events that accumulate during the neoplastic process. The insights gleamed from these genomic profiling has been instrumental to advancing therapeutic strategy. This chapter will aim to review the existing data with regards to chromosomal aberration, mutations, non-doing sequences, over-expressed mRNA, miRNA dysregulation and will explore the opportunities for major therapeutic developments in the cancer gemonic era.

2. Chromosomal aberration

Chromosomal aberration refers to an abnormality in the structure or number of chromosomal content of a cell. Increasingly, cancer is recognized as a heterogeneous collection of diseases whose initiation and progression are prompted by the aberrant function of genes that govern DNA repair, genome stability, cell proliferation, cell death, adhesion, invasion, angiogenesis in complex cell and tissue microenvironment [11, 12]. In addition to high-resolution chromosome banding and advanced chromosomal imaging technologies, chromosome aberrations in cancer cells can be analyzed with an increasing number of large-scale, comprehensive genomic and molecular genetic technologies. These growing technologies include fluorescence in situ hybridization (FISH) [13, 14], spectral karyotyping (SKY) [13], comparative genomic hybrizidation (CGH) [15, 16], and other high-throughput methods that detects loss of heterzygosity (LOH) [17, 18], in cancer cells such as a new single nucleotide polymorphism arrays (SNP Chips**)** [19] that detect comprehensive genome-wide copy number changes. With the use of comprehensive molecular technologies, the discovery of the recurrent chromosomal aberrations in cancer is proceeding at a very promising pace. To date, glioblastoma has been subjected to the most extensive genomic profiling of any cancer [20]. Studies carried out over the past three decades suggest that glioblastomas, like other cancers, arise secondary to the accumulation of genetic alterations. These alterations can present as epigenetic modifications, point mutations, translocations, amplifications, or deletions, and modify gene function in ways that dysregulate cellular signaling pathways leading to the cancer phenotype [11, 21]. While the exact number and nature of genetic alterations and deregulated signaling pathways required for tumorigenesis remains an issue of debate, [9] it is now well understood that central nervous system (CNS) carcinogenesis requires multiple disruptions to the normal cellular circuitry [22, 23].

Amongst chromosomal aberrations, amplifications and deletions can be distinguished when considering glioblastoma genesis [24]. Conversely, the reports of incidental translocation are rare in glioblastoma [25]. Thus we will mainly focus our review on chromosomal aberrations that present as amplification or deletion and discuss their contribution in the development of glioblastoma.

2.1. Amplification

Amplification of the epidermal growth factor receptor (EGFR) gene is a distinguishing feature in primary glioblastoma [26-28] Moreover, it is now evident that the type of genetic alterations involving EGFR in glioblastoma are distinct from those observed in other EGFR-altered cancers, such as non-small-cell lung cancer (NSCLC). In glioma, focal EGFR amplification occurs at an extremely high level (>20 copies) [20]. Focal (limited to a few Mb) and broader (from several Mbs to entire chromosomes) copy number alterations (CNAs) that include the EGFR gene may have different molecular consequences [27]. Focal amplification of EGFR correlates with EGFR over-expression or mutations and deletions in the EGFR gene, and subsequent activation of the PI3K/AKT pathway [27, 29]**.** Up-regulated

PI3K/ AKT signaling has been associated with poor prognosis [30]. Evidence of RTK/RAS/PI3K activation has been reported in 88% of tumors, including contributions from unexpected mutations or deletions in NF1 (18%) and PIK3R1, which encodes the p85a regulatory subunit of PIK3CA [20].

Furthermore, amplification of the entire chromosome 7 containing EGFR, MET [22] and its ligand HGF has been found to correlate with activation of the MET axis [20, 27]. EGFR amplification is reported to appear as double minutes (small fragments of extra-chromosomal DNA), and extra copies of EGFR have also been found inserted into different loci on chromosome 7 [31]. Additionally ~50% of EGFR-amplified cells harbor the EGFRvIII mutant, which is an intragenic gene rearrangement generated by an in-frame deletion of exons 2–7 that encode part of the extracellular region [20]. Remarkably, gain of chromosome 7 and amplification of EGFR have been found more frequently in short-term survivors [26, 32], however to date EGFR alterations are not thought to be of prognostic importance in glioblastoma [28, 32, 33].

Amplification of 12q13-15, where the oncogenes CDK4 and MDM2 are located, results in the disruption of both the retinoblastoma (RB) and p53 pathways [22, 27, 34, 35] Specifically, p53 signaling pathway has been reported to be impaired in 87% of the samples through CDKN2A deletion (49%), MDM2 (14%) and MDM4 (7%) amplification, and mutation and deletion of TP53 (35%) [20]. Pathway inactivating mutations in the RB pathway were described in glioblastomas prior to the large-scale genomic efforts [23, 36, 37] and the TCGA validated these results and demonstrated that mutations and gene amplifications disrupting RB function are found in approximately 68–80% of glioblastomas, signifying the critical importance of evading anti-growth signals [21]. RB signaling has been reported to be impaired in 78% of the samples through CDKN2 family deletion; amplification of CDK4 (18%), CDK6 (1%), and CCND2 (2%); and mutation or deletion of RB1 (11%) [20]. Additionally, Genome-Wide Association Studies (GWAS) revealed that single nucleotide polymorphisms (SNPs) in the CDKN2A and CDKN2B have been identified as risk factors for glioma growth [21] [38, 39]. Moreover, the genes encoding the receptor tyrosine kinases KIT, KDR, and PDGFRA, adjacently located on chromosome 4q12, are frequently found to be (co)amplified [40]. Nearly 30% of human gliomas show expression patterns that are correlated with PDGFR signaling [41]. For instance, PDGFRA amplification is found in 15% of all tumors [30, 42]. Of those PDGFRA amplified tumors harboring gene amplification, 40% harbor an intragenic deletion, termed PDGFRAD8, 9 [43], in which an in-frame deletion of 243 base pairs (bp) of exons 8 and 9 leads to a truncated extracellular domain [44]. Point mutations in PDGFRA are associated with amplification but, unlike EGFR, happen rarely. Elevated AKT phosphorylation has been observed in up to 85% of glioblastoma cell lines and patient samples [45]. RTK-independent activation of this pathway in glioblastoma can occur via mutation or amplification of PIK3CA (p110a) [46, 47], and PIK3CD (p110d) is also overexpressed in some gliomas [48]. Other amplified regions containing oncogenes, for example AKT3 [22, 49] and CCND2 [22, 27].

Over-expression of c-Myc is frequently observed in different tumor types, including glioblastoma, and usually results from chromosome translocation involving the c-Myc genes

in addition to gene amplification [50]. In a study it was reported that during multistep carcinogenesis using fibroblast lineages transfected with SV40 LT, expression levels of c-Myc and Sp1 associate with the levels of telomerase activity in different stages of transformation [51]. Transcriptional regulation of hTERT is thought to be the chief mechanism of telomerase regulation. Cooperative action of c-Myc and Sp1 is required for full activation of hTERT promoter. Sp1 is also a key molecule that binds to GC-rich sites on the core promoter and activates hTERT transcription [51]. In the core promoter, multiple E-boxes and Sp1 binding sites are located. C-Myc binds to these E-boxes through heterodimer formation with Max proteins and activates transcription of hTERT [52, 53]. This is a direct effect of c-*myc* that does not require *de novo* protein synthesis. Mad proteins are antagonists of c-Myc and switching from Myc/Max binding to Mad/Max binding decreases promoter activity of hTERT [51, 54-56]. Thus, up-regulation of these critical transcription factors may, at least in part, be involved in telomerase activation during carcinogenesis [57].

Amplified Region	Gene of Interest	References
1q	AKT3	[22, 49]
3q	PIK3CA	[22, 23, 27]
4q	PDGFR	[22, 34]
7p	EGFR, MET, HGF, CDK6	[22, 23, 27, 34, 35]
8q	c-MYC	[50]
12q	CDK4, MDM2	[22, 27, 35]

Table 1. Genes frequently identified to be amplified in glioblastoma

2.2. Deletions

Loss of heterozygosity LOH of chromosome 10q is the most common genomic alteration found in both primary and secondary glioblastomas [28, 35] and is associated with poor prognosis [26, 28]**.** Different regions are frequently lost at chromosome 10, including the regions containing PTEN, MGMT [28, 58], and ANXA7, an EGFR inhibitor [59]. PTEN directly antagonizes PI3K signaling and is one of the most frequently altered genes in cancer. It undergoes genomic loss, mutation, or epigenetic inactivation in 40%–50% of gliomas, resulting in high levels of PI3K activity and downstream signaling [60]. In addition, AKT activation due to PTEN loss likely contributes to RTK inhibitor insensitivity in glioblastoma [29, 61]. Another frequently deleted inhibitor of EGFR signaling is NFKBIA, which is located on chromosome 14; this deletion is also linked to poor survival [62]**.** Furthermore, loss of chromosome 9p, which contains a variety of tumor-suppressor genes, including CDKN2A, CDKN2B, and PTPRD, is frequently seen [28, 34, 63]**,** especially in short-term survivors [26, 32]**.** CDKN2A and CDKN2B encode three important cell cycle proteins, p14ARF and p16INK4A, and p15INK4B [26-28, 34, 64]**,** which are involved in the RB and P53 pathways. Deletion of CDKN2A and CDKN2B is often accompanied by deletion of CDKN2C on chromosome 1p32, which encodes another cell cycle protein p18INK4C [64]. LOH of chromosome 1p is found in both primary and secondary glioblastomas [65]. Longstanding hypothesis about the location of tumor suppressor gene at 1p has recently

been advanced by identification of the suggested candidate genes CIC and FUPB1 [66]. Co-deletion of 1p and 19q is frequently seen in oligodendrogliomas and is, in those, associated with prolonged survival [32] and translocations [67]. Although this co-deletion has been observed in glioblastomas, no similar association has been identified elsewhere. Isolated LOH 19q is frequently observed in secondary glioblastoma [26, 65] and may be a marker of longer survival [26]. Moreover >50% of oligodendrogliomas has been reported to display loss of heterozygosity (LOH) at chromosomes 1p and 19q [68], although the targets of these deletions are still unclear.

Frequent allelic losses on 22q indicating the presence of tumor suppressor genes have been found in primary and secondary glioblastomas [69]. LOH of 22q identified two sites of minimally deleted regions at 22q12.3–13.2 and 22q13.31 in primary glioblastomas and in most of the secondary glioblastomas. The affected shared deletion of 22q12.3 is the region in which the human tissue inhibitor of metalloproteinases-3 (TIMP-3) is located. As its name implies, expression of TIMP-3 inhibits metalloprotease activity and impair glioblastoma migration and invasiveness [70]. Expectedly, deletion of TIMP-3 enhances glioblastoma invasiveness [69].

It is important to note that the various deletions and amplifications do not exist in isolation. For instance, NFKBIA deletions and EGFR amplifications are essentially mutually exclusive events, suggesting that these events serve redundant functions in glioblastoma pathogenesis [62]. Systematic analysis of the patterns of co-occurrence of the various deletions and amplifications revealed genomic regions with synergistic tumor-promoting relationships [71]. Analysis of the general patterns of co-occurring and mutually exclusive regions in glioblastomas suggests common pathways that are disrupted during carcinogenesis. Targeting these pathways in the context of the genetic landscape of the glioblastoma constitutes one therapeutic strategy.

Deleted Region	Gene of Interest	References
9p	CDKN2A, 2B	[22, 27, 35]
10q	PTEN, MGMT, ANXA7	[22, 23, 34, 35]
13q	RB	[22, 34]
17p	P53, NF1	[22, 23, 34]
19q	BAX	[34, 65]
22q	TIMP3	[69]

Table 2. Genes frequently identified to be deleted in glioblastoma

3. Mutations

The abnormal behaviors demonstrated by cancer cells are thought to be the result of a series of mutations in key regulatory genes. A detailed understanding of the genomic lesions underlying cancer will facilitate the identification of the cellular pathways and networks perturbed by genomic mutations, improve cancer diagnosis through molecular classification, enhance the selection of therapeutic targets for drug development, promote

the development of faster and more efficient clinical trials using agents targeted to specific genomic abnormalities, and create markers for early detection and prevention. Results from the genomic profiling efforts and a number of studies over the past three decades have revealed that nearly all glioblastomas harbor activating mutations in genes that play instrumental role in growth signaling cascades, evading apoptosis, insensitivity to antigrowth signals. In addition to amplifications and deletions, genes implicated in glioblastoma can be affected by somatic mutations. Point mutations include base substitutions, deletions, or insertions in coding regions and splice sites. Large-scale mutation analysis has identified mutations activating oncogenes and others inactivating tumor-suppressor genes in glioblastoma.

It was previously thought that glioblastoma arises from the acquisition of a defined set of mutations that occur in a particular temporal order. This model is largely grounded on the framework established in colon cancer, where a series of genetic alterations characterizes different phases of neoplastic progression [72]. This hypothesis is supported by the observation that Grade II astrocytomas typically harbor mutations in p53; Grade III astrocytomas harbor activating mutations/amplifications of CDKN2A (p16Ink4a); and Grade IV astrocytomas harbor mutations in PTEN and EGFR [73]**.** This data was interpreted to suggest that glioblastoma results from sequential inactivation of the p53, RB, and RTK/PI3K axes. While such a paradigm may hold true for a subset of the secondary glioblastomas, the picture emerging from the genomic characterization of primary glioblastomas reveals a much more dynamic process [22, 23]. The profile of somatic mutations in different glioblastomas is highly variable. These results suggest that most glioblastomas, primary or secondary, evolve along a multitude of pathways in response to differing selective pressures to achieve the phenotypes described by Hanahan and Weinberg [74].

Aberrant centrosome behavior, such as centrosome amplification, has been associated with mutation of *TP53* and has been proposed as a primary source of genetic instability in human tumors. Mutations in ''common'' cancer genes, for example TP53 and PTEN, are very frequent in glioblastomas, but are not of prognostic importance [22, 23, 28, 32, 33, 75]. On the other hand PTEN loss has been shown clinically to confer resistance to EGFR inhibitors in patients harboring EGFRvIII expressing glioblastoma in part due to its activation of downstream AKT [29, 76] as well as loss of its RTK degradation function [76].

There are several lines of evidence that point to the importance of the p53 axis in glioblastoma pathogenesis. There is a body of literature associating p53 pathway inactivation to glioblastoma genesis [37, 77]. It must be noted that these studies implicate p53 pathway inactivation only in a subset of glioblastomas. The TCGA effort and the effort by Parsons et al. [22, 23] enhanced the literature by demonstrating that the p53 axis is more broadly impaired in glioblastomas than previously thought. Mutations that inactivate this axis are found in greater than 70% of all glioblastoma specimens as reported by both studies. This understanding has led to more accurate modeling of glioblastoma by combined inactivation of p53 and PTEN [78]**.**

There are a number of mutations that are thought be glioblastoma specific, even though they may be seen in only a subgroup of tumor cells. The EGFRvIII mutant lacks 267 amino acids in the extracellular part, resulting in a constitutively activated receptor that no longer requires its ligand EGF to signal downstream [79]. Despite the well-recognized proproliferative functions of EGFRvIII, its expression in human glioblastoma is heterogeneous and is most often observed only in a subpopulation of cells [80]. Recent observations support a model of functional heterogeneity in which a minority of EGFRvIII-expressing cells not only drive their own intrinsic growth, but also potentiate the proliferation of adjacent wild-type EGFR-expressing cells in a paracrine fashion through the cytokine co-receptor gp130 [81]**.** EGFRvIII expression may be linked to differentiation and/or development. EGFR point mutations have also been identified in glioblastoma, in the extracellular domain, whereas they are predominantly found in the kinase domain in other tumor types, such as lung cancer [82]. *EGFR* mutations have recently been identified as clinically significant, due to their association with striking responses in subsets of patients treated with targeted therapeutic agents. [83, 84].

The PI3K signaling pathway is dysregulated in many cancers [85], including glioblastomas. A number of investigations have reported activating mutations in the RTK–PI3K pathway [43, 86], validating the importance of this pathway in glioblastoma pathogenesis. Mutations in PIK3CA and PIK3R1, coding for the PI3K catalytic subunit p110a and regulatory subunit P85a, have been described [22, 23]. RTK-independent activation of this pathway in glioblastoma can occur via mutation of PIK3CA (p110a) [46, 47] or through recurrent mutations in the gene encoding the p85a regulatory subunit PIK3R1. This will likely drive PIK3CA activation through decreased SH2 domain-mediated inhibition [87]. In the TCGA report [22] activating mutations in the RTK–PI3K pathways are reported in 88% of the 206 glioblastomas sequenced.

Although mutations in the RAS genes constitute a fairly rare phenomenon in glioblastoma (>5%) [88], inactivating mutations and deletions have been identified in their inhibitory tumor suppressor gene NF1 [22]**.** The protein encoded by neurofibromatosis 1 (NF1) functions to catalyze the exchange of GTP for GDP in Ras - preventing cell proliferation. While it is reported that NF1 patients are predisposed to gliomagenesis [89], inactivating mutations in NF1 was not discovered in glioblastoma until recently [22, 23, 90, 91]. The TCGA results indicated that approximately 20% of glioblastomas harbor loss of function mutations in NF1 [22, 23] and more significantly, mutations in NF1 appear to define a particular subtype of glioblastoma.

The majority of malignant brain tumors, including glioblastoma, demonstrate inactivating mutations in either the p53 and/or retinoblastoma (RB) pathways [92-95]. In addition to their adverse cellular functions, these two pathways are most directly involved in cell cycling regulations during times of cell repair or cell growth.

The *TP53* tumor suppressor gene, located on 17p13, is frequently mutated or deleted in gliomas [96, 97]. P53 is a short-lived transcription factor that can execute diverse cellular programs, such as cell cycle arrest, DNA repair, apoptosis, autophagy, differentiation,

senescence and self- renewal [98, 99]. It facilitates DNA repair by halting the cell cycle for repair enzymes to work, or if the damage is too great, it induces cell death. The retinoblastoma (Rb, 13q14) pathway is also a key cell cycle regulatory complex at the G1 checkpoint. *CDKN2A,* located on 9p21 and deleted in many cancers, encodes the p16 protein, a key inhibitor of the cell cycle via Rb pathway signaling. Homozygous deletion of p16 has been reported to be associated with WHO grade III or IV gliomas [7, 100]. Gliomas often display mutations in the ARF- MDM2-p53 and p16INK4A-CDK4-RB tumor suppressor pathways [101, 102]. Primary glioblastoma often exhibits loss of the INK4A/ARF tumor suppressor gene locus along with PTEN mutation and EGFR amplification/mutation, and secondary glioblastoma shows frequent mutations of TP53 [58].

The relevance of p53 to the treatment and outcome of patients with high-grade glioma has remained controversial. Some studies have shown that p53 status, assayed either by expression or mutation analysis, is correlated with relatively good outcome [103, 104], while others have demonstrated no prognostic impact in anaplastic gliomas and GBM [105, 106]. Also, *MDM2* amplification, although infrequent, has been shown by some to be predictive of poor outcome [103, 107], whereas others have observed no prognostic value [108]. P53 status might cooperate with other prognostic variables; for example, *TP53* mutation has been linked to low *MGMT* mRNA expression [109], although this does not correlate with *MGMT* promoter methylation [110]. Loss of *CDKN2A, CDKN2B,* or *RB* or *CDK4* amplification, disrupting the Rb pathway, has been shown in anaplastic astrocytoma to associate with decreased survival [111, 112]. Conversely, p16 appears to be associated with improved survival in patients treated with chemotherapy and radiation [113]. Overall, it appears that the prognostic impact of p53 and Rb aberrations is at best marginal.

Comprehensive analysis of genomic data in glioblastoma revealed recurrent mutations in the R132 residue of isocitrate dehydrogenase 1 (IDH1) and is involved in energy metabolism [23]. IDH1/2 is mutated in grade II and III gliomas as well as the secondary glioblastomas that arise from prior low-grade tumors, with most mutations found in the IDH1 gene. IDH1 mutations have been predominantly identified in secondary glioblastomas and low-grade gliomas, with mutations in more than 70% of cases [23, 114-118]. Patients with IDH1 mutated primary glioblastomas are generally younger and have longer median survival and wild-type EGFR. Because these are characteristics of secondary glioblastomas, it is hypothesized that these are in fact secondary glioblastomas for which no histological evidence of evolution from a less malignant glioma is found. Significantly, these mutations usually occur at conserved residues and are virtually never homozygous. While only 3%–7% of primary glioblastomas harbor IDH1 mutations, the majority (50%–80%) of secondary glioblastomas express mutant IDH1. Thus, IDH1 could be used to differentiate primary from secondary glioblastomas [116]. In addition, 3% of the tumors that express wild-type IDH1 were found to express IDH2 R172 mutations [117-120], although this mutation in IDH2 has only been documented in a single glioblastoma in the literature [121].

Studies on the downstream biological effects of IDH1/2 mutation expression have focused largely on the inhibition of α-KG-dependent dioxygenases by 2-HG, as IDH mutations result in a novel function to catalyze α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG)

[122]. The wild-type IDH1 normally functions as a homodimer that converts isocitrate to α-ketoglutarate [120]. Biochemical depiction of the R132 mutated IDH1 revealed that it functions to inhibit the process. Thus, glioblastoma harboring the R132 IDH1 mutation harbor decreased levels of α-KG. It is imperative to note that α-KG dependent dioxygenases is a diverse group of enzymes controls a broad range of physiological processes, including hypoxic sensing, histone demethylation, demethylation of hypermethylated DNA, fatty acid metabolism, and collagen modification, among others [123]. Several studies have provided evidence to demonstrate that several of these functions are influenced by IDH1/2 mutation expression.

Mutational and epigenetic profiling of patients specimen has revealed that IDH1 mutations closely associated with a specific hyper-methylation signature. The hyper-methylation state may be caused in part by the 2-HG-mediated inhibition of the α-KG-dependent TET2 enzyme [124, 125]; the resultant decrease in 5-hydroxymethylcytosine was also observed in glioblastoma specimens [124]. Moreover, expression of IDH1 mutations is thought to induce global DNA hyper-methylation [126]. Thus it is suggested that IDH1 mutations may lead to dysregulated epigenetic processes. 2-HG inhibits histone demethylases and TET 5-methylcytosine hydroxylases, thought to be involved in epigenetic control. This suggests that mutations in IDH1 change the expression of a potentially large number of genes [124].

Most lower-grade gliomas harbor IDH1 mutations; although grade I pilocytic astrocytomas usually express wild-type IDH1; 60%–80% of grade II and III astrocytomas, oligodendrogliomas, and oligoastrocytomas express mutant IDH1, with the R132H mutation representing the majority of mutations observed. Given that mutations in IDH1 are an early event in gliomagenesis [127], this may suggest widespread modification of epigenetic regulator as the key mechanism in gliomagenesis in IDH1 mutated tumors. Furthermore, it might explain the extensive and fundamental differences between mutated and wild-type IDH1 glioblastoma. It has been reported that global expression profiles of IDH1 mutant glioblastomas more closely resembled lineage-committed neural precursors, whereas wild-type counterparts appear to resemble neural stem cells [128].

Independent glioblastoma studies have pointed to IDH1 mutations as an objective positive prognostic marker [23, 114, 115, 120]. Reports of the association between IDH1 mutations and favorable prognosis hold promise for biomarker development [23, 42, 120], although these correlations await validation in prospective clinical trials. Thorough understanding of mutant IDH biology and the mutant status of the IDH1/2 genes may serve as a key prognostic indicator. Specifically, patients with anaplastic astrocytoma [23, 115, 120, 121] and glioblastoma harboring mutant IDH1 demonstrate a significantly longer overall survival compared with wild-type IDH1 counterparts and are younger at presentation. Similar survival benefit has also been observed in grade II gliomas. [115] Furthermore, a comprehensive genomic and clinical analysis of glioblastomas harboring mutant and wild-type IDH1 suggests that, while histo-pathologically similar, these tumors may represent disease processes far more unique than has been appreciated. Specifically, IDH1 mutant tumors display less contrast enhancement, less peritumoral edema, larger initial size, greater cystic components, and a greater likelihood of frontal lobe involvement compared with wild-type tumors [128].

A frequently encountered critique of genomic sequencing effort involves the following. The first generation sequencing used to characterize the glioblastoma landscape captures the most prevalent mutations. They did not analyze the deeper heterogeneity of low prevalence mutations that have been found in several tumor types, including colon cancer [129]. Efforts to examine whether such sub-clonal diversity exist in glioblastoms using highly sensitive techniques [130] have not identified the presence of low-prevalence mutations. These results suggest that clonal expansion of select mutation in glioblastoma constitute a major mechanism of tumor expansion and that random mutagenesis through mutator phenotype does not contribute significantly to glioblastoma pathogenesis. The insights gained from the TCGA and other sequencing efforts should be viewed in this light.

4. Non-coding DNA sequences

While the identification of nucleotide alterations within the coding sequence of protooncogenes or tumor suppressor genes has significantly contributed to our understanding of carcinogenesis, there is an emerging appreciation that alterations in non-coding sequences similarly contribute to development of cancer [131]. Non-coding DNA describes components of DNA arrangements that do not participate in the coding of protein sequences. These DNA sequences may present in different forms including non-coding functional RNA, cis- and trans-regulatory elements, introns, pseudogenes, repeat sequences, transposons, and telomeres. A notable example involves the regulation of gene transcription by reversible modification of gene promoter regions a phenomenon often referred to as 'epigenetic regulation' [132]. The term 'epigenetic regulation' describes the phenomenon in which heritable changes in gene expression can occur in the absence of changes in the DNA sequences encoding for gene function. Understanding the concept that non-coding sequences play critical roles in glioblastoma pathogenesis and resistance to chemotherapy offers novel strategies for biomarker development and therapy.

The mechanism underlying epigenetic involves cytosine methylation [133] or histone modifications that, in turn, modulate the accessibility of gene promoter regions to transcriptional factors [134]. Cytosine methylation often occurs in the context of CpG di-nucleotide repeats, or CpG islands [133]. Thus promoters that harbor heavily methylated CpG islands are typically transcriptionally silenced. There are two types of promoter methylation that are particularly pertinent to glioblastoma therapy: methylation in the promoter region of the DNA repair gene, methyl-guanine methyl transferase (MGMT) and the glioma-CpG island methylator (G-CIMP) phenotype [135].

MGMT encodes an enzyme that removes alkyl adducts at the O6 position of guanine [136]. Because alkyl modification at this position is highly toxic and constitutes the primary mechanism for the tumoricidal activity of the chemotherapeutic agent TMZ, MGMT expression level correlates well with TMZ response in patients with glioblastoma [137]. The human MGMT gene possesses a CpG island that spans approximately 1000 bases around the transcriptional start site. Detailed analysis of this region revealed 108 CpG sites [138] that are methylated. Methylation of a subset of these CpGs has been associated with

transcriptional silencing of MGMT [139, 140] and is associated with improved clinical outcome in patients with glioblastoma receiving TMZ therapy. Interestingly, MGMT promoter methylation is also associated with improved survival in patients who did not receive TMZ therapy [141, 142]. While the mechanism underlying this observation remains unclear, it seems likely that MGMT may participate in detoxifying the accumulation of endogenous DNA damage that is typically associated with the oncogenic state [143]. Glioblastoma cells accumulate endogenous DNA damage in the absence of DNA damaging agents [143]. These endogenous DNA damages are not unlike those induce by temozolomide or radiation in that they could trigger cell death if unrepaired. Thus, tumors with high levels of MGMT may grow more robustly since MGMT is capable of detoxifying these endogenous DNA damages. If the tumor cells grow more robustly, the patient will survive for a shorter duration. In contrast, the glioblastoma cells with low MGMT may be more susceptible to the deleterious effects of the endogenous DNA damages. These tumors may grow less robustly, resulting in longer patient survival.

The G-CIMP phenotype refers to the observation that a subset of glioblastomas exhibits concerted CpG island methylation at a large number of loci [144]. Since genes required for tumour growth are located at many of these loci, glioblastomas harboring the G-CIMP phenotype tend to be more benign. Correspondingly, patients with G-CIMP glioblastomas experienced significantly improved outcome. Understanding the concept that the patterns of CpG island methylation directly impact outcomes in patients with glioblastoma open the door to therapeutic strategies aimed at enhancing promoter methylation at select promoter loci. Importantly, recent studies suggest that promoter methylation at distinct loci may be affected by specific chromatin-modulating factors [135, 145].

While much of cellular DNA has no known biological function, many types of non-coding DNA sequences do have recognized biological functions, including the transcriptional and translational regulation of protein-coding sequences. These governing functions may include genetic switches, regulation of gene expression, transcription factors, operators, enhancers, promoters, and insulators [146-148]. Genome-wide association (GWA's) studies have uncovered a large number of cancer susceptibility regions that do not overlap protein-coding genes but rather map to non-coding intervals [132, 135]. The concept that non-coding DNA sequences regulate gene function and impact carcinogenesis has significantly expanded the repertoire of strategies available for glioblastoma therapeutics [135]. Integrating the biology of non-coding sequences in the context of mutational profile is critical in understanding tumor physiology and meaningful therapeutic development.

5. Over-expressed mRNA

Over-expression or under-expression of genes in glioblastoma compared with that in a normal brain or in low-grade gliomas may serve as an indication of genes that are involved in gliomagenesis [24]. While glioblastoma has been conceptualized as a single disease, it is widely appreciated that the term captures significant histologic heterogeneity. This heterogeneity suggests distinct subtypes with differing physiologic states that are captured

under the umbrella term ''glioblastoma'' [21]. In fact, the genome-wide analysis of mRNA expression to identify molecular subclasses (Golub et al. 1999) has led to a fundamental shift in our understanding of glioblastoma subtypes. In fact, the identification of multiple subtypes within glioblastoma has highlighted the heterogeneity of diseases that are in the same group based on the WHO histo-pathological grade.

Primary and secondary glioblastoma subtypes are histo-pathologically indistinguishable, but differences can be demonstrated by molecular markers at the epigenetic [69], genetic [28, 35, 58], expression [149], and proteomic [150] levels. Primary glioblastomas have a greater prevalence of EGFR alterations, MDM2 duplications, PTEN mutations, and homozygous deletions of CDKN2A [28, 58]. MET amplification [35], over-expression of PDGFRA, and mutations in IDH1 and TP53 are more prevalent in secondary glioblastomas [23, 29, 58, 75, 114, 116, 118]. Moreover, the large-scale analysis has revealed the highly structured nature of glioma transcriptome and has shown correlation of tumor histology and molecular alterations with patient outcome [10, 24, 42]. While expression profiling of glioblastoma has been widely used, two fundamental studies have provided the groundwork for the classification of glioblastoma subtypes [30, 42]. The first subtype initially reported by Phillips et al. [30] and subsequently confirmed by the TCGA mRNA [42] and microRNA profiling [151]. The transcript signature resembles those of neuro-blasts and oligodendrocytes derived from fetal and adult brain cells [30]. The subtype harbors transcriptomal and clinical features that emulate those previously classified as secondary glioblastomas. Molecularly, proneural glioblastomas harbor mutations classically associated with the secondary glioblastomas [42]**.** Hence, grade II and III gliomas harbor transcriptomal signatures most reminiscent of the proneural subtype [30]. Clinically, this subtype typically affects younger patients, is associated with improved overall survival [30], and responds poorly to concurrent radiation/temozolomide treatment upon disease progression [42].

The second subtype that has emerged is characterized by a gene expression signature that illustrates those observed in the neural stem cells of the forebrain [30], cultured astroglial cells [152], and tissue of mesenchymal origin [30]. Thus, the subtype is termed ''mesenchymal'' for the latter correlation. Similar to the proneural subtype, this second subtype was initially identified by Phillips et al. [30] and subsequently confirmed by the TCGA [42]. This subtype is highly enriched for mutations inactivating NF1, suggesting a common genetic etiology. The mesenchymal signature appear driven a common transcriptional network, as expression of two key critical factors (STAT3 and CEBPb) enhance tumor aggressiveness in murine models [153].

Benefiting from unsupervised hierarchical clustering analysis, Verhaak et al. (2010) classified 200 TCGA glioblastoma samples into four subtypes, which were subsequently validated using previously published data from 260 independent samples. Large-scale expression studies are validated by reverse transcription (RT)-PCR for individual genes. Bioinformatics analysis revealed that three of the four subtypes were found to harbor distinct molecular aberrations. In particular, the proneural subtype was enriched for amplifications of PDGFRA, CDK6, CDK4, and MET; 11 out of 12 IDH1 mutations found in

the TCGA samples; PIK3CA/ PIK3R1mutations; and mutation or LOH of TP53. While the mesenchymal subtype carries mutations and/or loss of NF1, TP53, and CDKN2A, the classical subtype shows amplification for EGFR and loss of PTEN. On the other hand, to date no distinguishing genetic alterations have been indicated to define the neural class from the other classes [20]. It is imperative to keep in mind that interpretations of these results are difficult due to methodological differences in profiling platforms, bioinformatic extrapolation, and specimen collection.

While the number of subtypes identified by the Verhaak et al. (2010) and Phillips et al. (2006) studies differs, the proneural and mesenchymal classifications identified using distinct methodologies and sample sets are the most robust and concordant [10]. For instance, both groups identified proneural class expression of DLL3 and OLIG2 and mesenchymal class expression of CD40 and CHI3L1/YKL-40, the latter of which appears to be a potential serum protein marker of prognosis in glioblastoma patients [154]. Both studies share the observation that patients afflicted with the mesenchymal subtype exhibit poorer clinical prognosis relative to the proneural subtype. A high level of expression of insulin-like growth factor binding proteins, for example IGFBP-2/3 [155], angiogenesic factors, such as vascular endothelial growth factor A (VEGFA) [156], and mesenchymal markers, like YKL-40/CHI3L1, are frequently seen in glioblastoma and have been associated with poor prognosis [157-159]. In contrast, NOTCH signaling genes, for example DLL3, are indicative of better survival [160].

Hence, the collection of data suggests at least two distinct subtypes that reflect essential biologic behavior [10, 30, 42] and have been validated by independent studies. In addition to promising improvement in the grading of glioblastoma, gene expression profiling has shown great promise in prognosis of this deadly tumor, as the genes represented in these subtypes could help to predict outcome in glioblastoma. For example, increased expression of mesenchymal genes such as CHI3L1/YKL-40 and LGALS3 combined with decreased expression of a proneural gene, OLIG2, are associated with typical short-termsurvival compared with longer-termsurvivors [161]. Additional studies have extended the utility of mRNA profiling by using computational network analysis to uncover the causal regulatory modules underlying particular transcriptomically defined subtypes. It is important to note that most of these subtypes have not been as rigorously validated as the proneural and the mesenchymal. The emerging literature suggests that the proneural and mesenchymal subtypes define the two poles in the spectrum of molecular glioblastoma physiology [10, 30, 42]. It remains unclear whether the other proposed subtypes constitute a ''forced fit'' of a set of truly heterogeneous biology, a gradation of phenotypes between the two extreme poles, or a genuine subtype whose biologic basis remains to be understood.

With genomics approaches, discoveries of common features of different types of tumor may lead to new therapeutic targets and drugs for other tumor types also. The discovery of overexpression of VEGFA and its correlation with poor prognosis in glioblastomas [156] led to trials with the angiogenesis inhibitor bevacizumab.

6. Micro-RNA (miRNA) dysregulation

Micro-RNAs (miRNA or miR) are a class of small non-coding RNAs, approximately 22 nucleotides long that are involved in post-transcriptional gene regulation [162]. Through imperfect pairing, miRNA's bind to untranslated regions of protein-coding mRNAs and function mainly as negative regulators of gene expression. Binding of miRNA often leads to mRNA degredation or inhibition of protein translation – resulting in suppression of the target proteins. A number of cellular processes are regulated by miRNAs including development, proliferation, and differentiation. Micro-RNAs play an important role in many different disorders, particularly in cancer [163]. Bioinformatic analysis predicts that a single miRNA can potentially regulate hundreds of target oncogenes or tumour suppressor proteins. The association of miRNA deregulation with pathogenesis and progression of malignant disease illustrates great potential of utilizing miRNAs as targets for therapeutic intervention. Thus, modulation of miRNA expression provides great hope for potential cancer therapy. Furthermore, since each miRNA may have more than one target, miRNA-based gene therapy offers the therapeutic appeal of targeting multiple gene networks that are controlled by a single miRNA [164]. Over 1000 miRNAs have been described in humans [165]. Bioinformatics analysis has recently revealed that miRNAs are differentially expressed in glioblastoma tissues compared to normal brain tissue [166-169]. For example, while primary glioblastomas and cell lines over-express miR-221 and miR-222, which are thought to target cell cyclin-dependent kinase inhibitors p27 and p57, set of brain-enriched miRNAs (miR-128, miR-181a, miR-181b, and miR-181c) show reduced expression [170, 171].

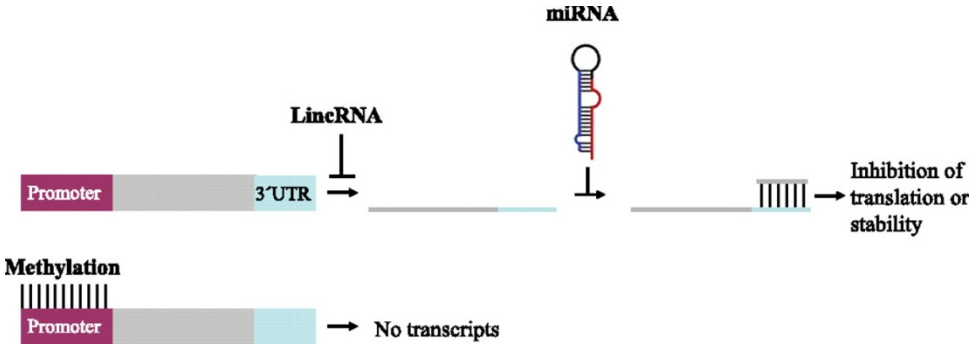

Figure 1. Gene regulation by non-coding RNAs. Figure is adapted with permission from reference [135].

Frequently up-regulated miRNAs are called onco-miRNAs and are thought to contribute to carcinogenesis. As an example miRNA-10b is known to be highly expressed in glioblastoma samples [170], suggesting an important role for miR-10b in glioblastoma tumorigenesis. Furthermore, a recent study revealed that miR-10b expression is inversely correlated with glioblastoma patient survival [172]. Notably, miR-10b was also found to be up-regulated in breast cancer, leukemia, and pancreatic cancer and promote tumor invasion and metastasis in breast cancer [173-175]. These results suggest that some miRNAs, such as miR-10b, may function as a global oncogene to trigger tumorigenesis in multiple tissues. Another example

of onco-miRNA in glioblastoma is miR-26a, which is thought to target PTEN [176]. PTEN has been reported to be down-regulated in 70% of human cancers, and there are several indications that it functions as a haplo-insufficient tumor suppressor gene [177]. PTEN expression is down-regulated by several different miRNAs, and it is thought that post-transcriptional regulation is an essential player in determining PTEN abundance in cancer cells. By targeting the tumor suppressor PTEN, overexpression of miR-26a facilitates tumorigenesis [168, 176]. Furthermore, miR-26 cooperates with oncogenes CDK4 and CENTG1, forming an onco-miRNA/oncogene cluster, targeting the RB, PI3K/AKT, and JNK pathways and increasing aggressiveness in glioblastoma [168]. Over-expressed oncogenic miRNAs may be targeted by antagomirs or miRNA sponges, because over-expression of the onco-miRNAs miR-26a, miR-196, and miR-451 has been correlated with poorer survival [167].

In contrast with the onco-miRNA's, frequently down-regulated miRNA's in glioblastoma are considered tumor-suppressor miRNA's. Reduced miR-128 expression in glioblastoma and consequent reduced cell proliferation *in vitro* and in xenografts [178]. Furthermore, miR-128 regulates the expression of the complex protein Bmi-1 through binding at the *BMI-1* 3'-UTR, resulting in decreased Bmi-1 and H3K27me3 levels. In GBM-derived neurosphere cells, miR-128 over-expression has been reported to block stem cell self-renewal, indicating that miR-128 can govern the stem cell-like capabilities of a subset of GBM cells [132]. Glioblastoma tumor tissue profiling has revealed that miRNA-124 is down-regulated in glioblastoma tissue [163, 170]. Notably, miR-124 is also frequently down-regulated in other cancers, such as medulloblastoma, hepatocellular carcinoma, and oral squamous carcinoma [179, 180], suggesting that it may function as a general tumor suppressor. Moreover, miRNA-137 and miRNA-451 exhibit reduced expression in malignant glioblastoma tissues relative to normal brain tissues [181, 182].

Despite advances in biomedical science, the prognosis of glioblastoma patients remains poor. Biomarkers for this disease are needed for early detection of tumor progression. Clinical significance of miRNA expression profiles in glioblastoma has not been explored extensively. Nevertheless, 16 candidate miRNAs have been described to associate with malignant behavior of gliomas (miR-196a, miR-15b, miR-105, miR-367, miR-184, miR-196b, miR-363, miR-504, miR-302b, miR-128b, miR-601, miR-21, miR-517c, miR-302d, miR-383, miR-135b). Among them, miR-196a and miR-196b indicated the highest level of significance) [183]. Both miRNAs showed increased expression levels in glioblastomas relative to anaplastic astrocytomas and normal brain tissues. Higher level of miR-196 transcript significantly correlated with poorer survival [167, 183]. Treatment of malignant gliomas remains one of the greatest challenges facing oncologists today through a frequent resistance to both chemo- and radiotherapeutic agents [184]. Important question for management of glioblastoma patients is the possibility of predicting therapeutic outcome. The miRNA expression profiles of glioblastoma tissues have shown association of miR-181b and miR-181c with response to concomitant chemoradiotherapy with temozolomide (RT/RMZ). MiR-181b and miR-181c were significantly down-regulated in glioblastoma tissue of patients who responded to RT/TMZ in comparison to patients with progressive

disease [183, 185]. In a recent study by Zhang et al. [186] genome-wide miRNA profiling of 82 glioblastomas demonstrated that miR-181d was inversely associated with patient overall survival and temozolomide (TMZ) treatment. Bioinformatics analysis of potential genes regulated by miR-181d revealed methyl-guanine-methyl-transferase (MGMT) as a downstream target. Together, these results suggest that miR-181d is a predictive biomarker for TMZ response and that its role is mediated, in part, by post-transcriptional regulation of MGMT.

The basic strategy of current miRNA-based treatment studies is either to antagonize the expression of target miRNAs with antisense technology or to restore or strengthen the function of given miRNAs to inhibit the expression of certain protein-coding gene. Unfortunately, several major challenges have to be addressed before the application of miRNA-based treatment. First, the multi-targeting nature of miRNAs gives the risk of unintended off-target effects that need to be carefully evaluated. Moreover, the expression of target gene may be governed by several different miRNAs, which may compromise the effect of miRNA-based treatment. Finally, there is still lack of miRNA delivery system with enough specificity and efficacy [183].

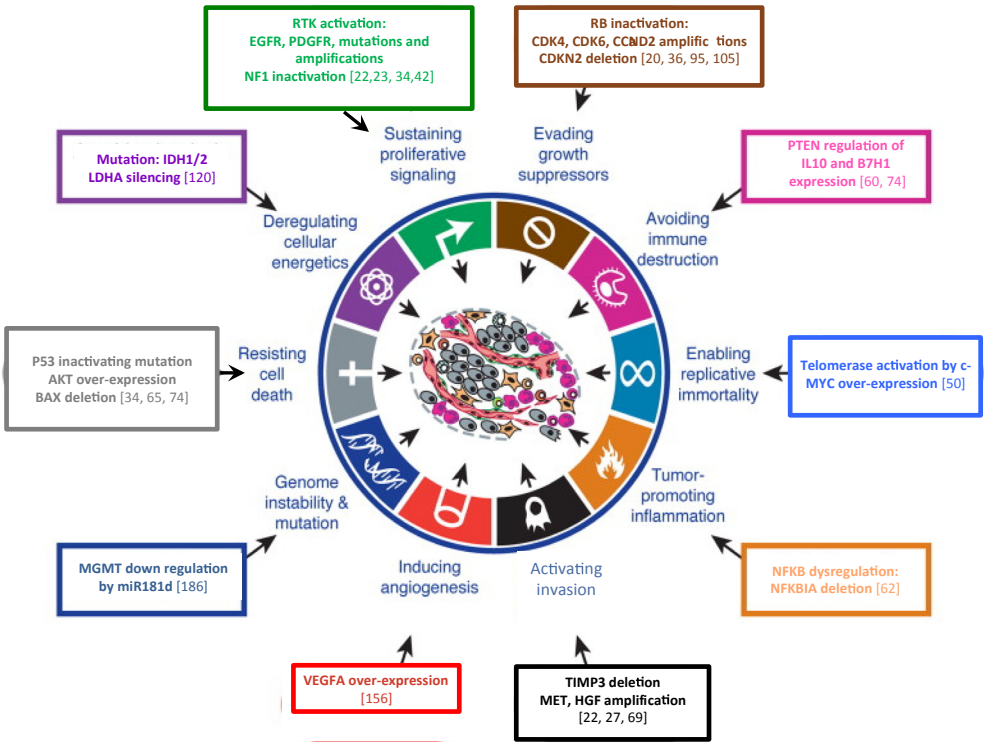

Figure 2. TCGA revealed genes that are known to contribute to the cancer phenotype, as proposed by Hanahan and Weinberg (2011). Figure is adapted with permission from reference [8].

7. Conclusion

In this chapter, we have reviewed and discussed key molecular participants glioblastoma, including chromosomal aberration, mutations, non-coding DNA sequences, over-expressed mRNA, and miRNA dysregulation. We placed our focus to explore the opportunities for major therapeutic developments in the cancer genomic era, where a more comprehensive mechanistic insight into glioblastoma pathogenesis and biology is arguably the most promising approach to discoveries of innovative treatment strategies.

Future development of tools for subtyping, biomarker development, and therapeutic strategies grounded in the genomic landscape of the particular glioblastoma will facilitate clinical trial designs. Ultimately, robust therapeutic gain can be achieved only when agents are directed toward the most vulnerable features inherent within the distinct physiologies of different glioblastoma.

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