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REVIEW

Molecular Heterogeneity of Glioblastoma and its Clinical Relevance

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Abstract Glioblastoma is the most common intracranial malignancy and constitutes about 50 % of all gliomas. Both inter-tumor and intra-tumor histological heterogeneity had been recognized by the early 1980-ies. Recent works using novel molecular platforms provided molecular definitions of these tumors. Based on comprehensive genomic sequence analyses, The Cancer Genome Atlas Research Network (TCGA) cataloged somatic mutations and recurrent copy number alterations in glioblastoma. Robust transcriptome and epigenome studies also revealed inter-tumor heterogeneity. Integration and cluster analyses of multi-dimensional genomic data lead to a new classification of glioblastoma tumors into subtypes with distinct biological features and clinical correlates. However, multiple observations also revealed tumor area-specific patterns of genomic imbalance. In addition, genetic alterations have been identified that were common to all areas analyzed and other alterations that were area specific. Analyses of intra-tumor transcriptome variations revealed that in more than half of the examined cases, fragments from the same tumor mass could be classified into at least two different glioblastoma molecular subgroups. Intra-tumor heterogeneity of molecular genetic profiles in glioblastoma may explain the difficulties encountered in the validation of oncologic biomarkers, and contribute to a biased selection of patients for single target therapies, treatment failure or drug resistance. In this paper, we summarize the currently available literature concerning inter- and intra-tumor molecular heterogeneity of glioblastomas, and call attention to the importance of this topic in relation to the growing efforts in routine molecular diagnostics and personalized therapy.

Keywords Glioblastoma · Inter-tumor heterogeneity · Intra-tumor heterogeneity · Molecular classification · Molecular heterogeneity · Primary brain tumor

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Introduction

Glioblastoma is the most common primary brain malignancy in adults and one of the most aggressive cancers. It accounts for 16 % of all primary brain tumors, and represents half of gliomas. The incidence of glioblastomas increases with age, and the rates are highest in the 75 to 84 years old age-group. The relative survival estimates for glioblastoma are quite low: less than 5 % of patients survive 5 years post-diagnosis [1]. It may involve any neuroanatomical structure, but is most commonly located in the cerebral hemispheres. Histologically, glioblastoma is a diffuse, grade IV glioma of the astrocytic lineage (WHO Classification of Tumors of the CNS, 4th edn, 2007) and is characterized by the presence of pleomorphic cells, mitotic activity, intravascular microthrombi, necrosis with or without cellular pseudopalisading and microvascular proliferation. Based on the presence or absence of a precursor lesion, two similar morphological subtypes are distinguished: primary glioblastoma is the most common type (~90 %) which arises de novo, without evidence of a precursor lesion, and typically occurs in older adults (>50 years), while secondary glioblastoma evolves from a pre-existent, lower-grade astrocytoma (WHO grades II or III) [2].

Histological studies establish that glioblastoma is an extremely heterogeneous tumor. Great variations may be observed in the types and sizes of cells (small anaplastic, large anaplastic cells, fibrillary astrocytes, pleiomorph astrocytes), the numbers of mitosis, and the distributions of cell density,

calcification, vascularization and necrosis [3]. A glioblastoma tumor may also include tumor cells of non-glial origin (e.g. primitive neuroectodermal tumor). This heterogeneity can be observed not only among tumors, but also within each tumor. Individual cancer cells tend to be similar to their immediate neighbors, while more distant cells typically have markedly different characteristics, which may reflect a clonal evolution and divergence within the tumor. The inter- and intra-tumor patterns of heterogeneity have different biological and diagnostic implications. In this paper, we chronologically survey data concerning these two different patterns of heterogeneity in glioblastomas, and highlight the importance of chromosomal, genomic, transcriptomic and epigenomic observations that may need to be considered in future diagnostics and therapy.

Inter-Tumor Heterogeneity

Early Chromosome Studies of Glioblastoma Heterogeneity

Shapiro et al., wrote in the 1980-ies that "increasing evidence, both experimental and in the clinic, suggests that some cancers are not homogeneous in their cell composition but are composed of heterogeneous cell types. This heterogeneity appears to contribute to the variable response to chemotherapy of the tumor as well as to variations in its cell surface markers, tumor antigens, growth rates, and its capacity to produce intra- and extracellular proteins." The authors karyotyped single-cell suspensions derived from 8 dissociated human gliomas. Two hundred and fifty or more metaphases were examined for each tumor to determine the probable number of cellular subpopulations present at the time of resection. Then, the investigators designated a subpopulation as a cellular representative of the resected tumor if 5 or more karyotypes with identical deviations were found. The number of subpopulations ranged from 3 to 21 per tumor [4]. In the 1990's and in the early 2000's, most of the heterogeneity studies focused on the chromosomal aberrations of glioblastoma, and on the different karyotypic subpopulations of tumor cells. Loeper et al. investigated whether or not the non-random chromosomal aberrations such as polysomy 7 and monosomy 10, merely reflected general chromosomal instability and a high overall incidence of mitotic errors by analyzing the frequency and distribution patterns of chromosomes known to be often numerically aberrant (7, 10) and chromosomes found to be numerically more stable in earlier in vitro karyotype studies (8, 12, 17, 18). The data indicated no apparent area-specificity in numerical aberrations of chromosomes 7, 8, 10, 12, 17, and 18, and there was no correlation with the local histomorphologic patterns [5]. The chromosomal heterogeneity of gliomas was tested by various techniques including karyotype analyses alone or combined with flow cytometry [6], DNA fingerprinting [7] and comparative genomic hybridization (CGH) [8]. The possibility that molecular classification may complement histopathological diagnosis was raised around this time. However, the molecular data had been insufficient and the conclusions equivocal [9]. Further studies were needed for the molecular characterization and classification of glioblastoma.

Molecular Approaches to Define Glioblastoma Heterogeneity (A-D)

A. *Identification of somatic mutations and copy number alterations*:

Initial molecular studies identified the following genetic events in human glioblastomas: (1) dysregulation of growth factor signaling via amplification and mutational activation of receptor tyrosine kinase (RTK) genes (e.g. the genes of the epidermal growth factor receptor – EGFR, platelet-derived growth factor receptor A - PDGFRA); (2) activation of the phosphatidylinositol-3-OH kinase (PI3K) pathway; and (3) inactivation of the p53 and retinoblastoma (RB) tumor suppressor pathways [10]. In 2005, The National Cancer Institute and the National Human Genome Research Institute launched a comprehensive project, The Cancer Genome Atlas (TCGA), to improve the understanding of the molecular basis of cancer. TCGA aimed to discover and catalogue major cancer-causing somatic alterations in large cohorts of human tumors through integrated multi-dimensional genomic sequence, transcriptome and epigenome analyses. The first cancer studied by TCGA was glioblastoma. The network analyzed complete sets of DNA, or genomes, of tumor samples obtained from 206 patients with glioblastoma. In addition to the previously recognized alterations, the TCGA studies revealed significantly recurrent focal alterations including homozygous deletions in genes of neurofibromatosis type-1 (NF1) and parkin RBR E3 ubiquitin protein ligase (PARK2), and amplifications in the gene of RAC-gamma serine/threonine protein kinase (AKT3). The abundance of protein-coding genes and non-coding micro-RNAs (miRNAs) was also assessed by transcript-specific and exon-specific probes on multiple platforms. The resultant integrated gene expression dataset showed that, 76 % of genes within recurrent copy number alterations have expression patterns that correlated with copy numbers. In addition, single-nucleotide polymorphism (SNP)-based analyses revealed copy-neutral loss of heterozygosity (LOH), with the most significant region being 17p containing TP53. The TCGA analysis identified a highly interconnected network of aberrations, including three major pathways: RTK signaling, and the p53 and RB tumor suppressor pathways. Deregulation of these three pathways appeared to be a core requirement for glioblastoma pathogenesis [11].

Parallel with the TCGA project, Parsons et al. analyzed 20,661 protein coding genes in 22 human glioblastoma samples by using sequencing. The candidate cancer genes (CAN-gene) included TP53, PTEN, CDKN2A, RB1, EGFR, NF1, PIK3CA, and PIK3R, which had been previously detected in gliomas. Further critical gene alterations were identified in the p53 pathway (TP53, MDM2, and MDM4), the RB pathway (RB1, CDK4, and CDKN2A), and the PI3K/PTEN pathway (PIK3CA, PIK3R1, PTEN, and IRS1). However, the newly discovered CAN-gene list included a number of individual genes that previously had not been linked to glioblastomas. The most frequently mutated gene was the isocitrate dehydrogenase-1 (IDH1) gene, which was most frequently detected in younger glioblastoma patients and in nearly all patients with secondary glioblastomas. Patients with IDH1 mutations had a significantly better prognosis, with a median overall survival of 3.8 years as compared to 1.1 years for patients with wild-type IDH1. Patients with IDH1 mutations also had a very high frequency of TP53 mutations and a very low frequency of mutations in other commonly altered glioblastoma genes [12]. These results were replicated by Verhaak et al.[13].

The importance of the TP53 and IDH network in glioblastoma was confirmed by another computational method called network analysis revealing interactions among aberrant genes and their transcripts. This approach identified genes frequently implicated in tumorigenesis (e.g., ATM, FGFR1, IDH1, MET, MSH6, NF1, RB1, and TP53) and revealed 1,001 genes with robust over- or underexpression in association with IDH1 mutation status [14]. The authors also found that 543 genes have robust over- or underexpression patterns associated with the spectrin repeat containing, nuclear envelop-1 (SYNE1) gene mutation status in glioblastoma. While SYNE1 mutations have been associated with lung, ovarian and colorectal cancers, it remains to be confirmed that SYNE1 has a role in glioblastoma and was not highlighted as a mere artifact of computational analysis [14].

Cerami et al. also used a network-based approach for identifying candidate oncogenic processes and key genes involved in pathogenesis [5]. In this study, the glioblastoma network was partitioned into network modules or clusters of network nodes joined together in tightly knit groups, between which there were only looser connections. The authors confirmed the observation that glioblastoma alterations tend to occur within specific functional modules. This approach was able to automatically identify the main p53, RB and PI3K signaling modules. While the TCGA approach identified eight genes as significantly mutated, in the network analysis seven of these genes appeared within the glioblastoma network, and all seven appeared within the two largest modules (RB1 module, PIK3R1 module). Interestingly, NF1 did not appear within the glioblastoma network [15].

In addition to the identification of the lists and networks of genomic alterations characteristic of glioblastoma subsets, the definition of functional correlates of mutations represents another level of complexity. Namely, a fundamental and open challenge in cancer genomics is the ability to distinguish “driver” from incidental “passenger” mutations. A driver mutation has a direct pathogenic role in oncogenesis, confers growth advantage on the cancer cell and is positively selected in the microenvironment of the tissue in which the cancer arose. However, a driver mutation is not necessarily (although it often is) required for the maintenance of the cancer but it must have been selected at some point along the lineage of cancer development. A passenger mutation is not selected, does not confer clonal growth advantage and therefore, does not contribute to cancer development [16, 17].

The above survey summarizes those major efforts in the characterization of the glioblastoma genome which led to the identification of somatic changes in well-known glioblastoma genes (e.g. EGFR, PTEN, IDH1, TP53 and NF1) and refined the list of putative driver genes with somatic mutations, but often without functional analyses.

Cerami et al. identified new candidate drivers, including AGAP2/CENTG1, a putative oncogene and an activator of the PI3K pathway, and three new modules (DCTN2, NUP107, and IFNAR1 module) of potential interest by network analysis [15]. AGAP2, also known as PIKE and CENTG1, is amplified in 19 % of the glioblastoma cases. PIKE-A, one of the three isoforms of AGAP2, specifically binds to active AKT [18]. The authors postulated that AGAP2 may represent an alternative mediator or additional means by which glioblastoma tumor cells activate the PI3K pathway and its downstream effects, including cell proliferation, inhibition of apoptosis, and tumor invasiveness. Evidence supports that disruption of the interaction between PIKE-A and AKT by small antagonist peptides significantly reduces glioblastoma cell proliferation, colony formation, migration and invasion, while also enhances the effects of the commonly used chemotherapeutic drugs (temozolomide, Carmustine-BCNU) at least in cell culture conditions [19].

Frattini et al. integrated somatic mutations from whole-exome sequencing with a copy number alteration (CNA) analysis [11]. This approach prioritizes the focality and magnitude of the genetic alterations in order to uncover candidate driver genes in glioblastoma. Somatically mutated genes were stratified into three groups: recurrently mutated genes without CNAs, genes in regions of focal and recurrent amplifications, and genes in regions of focal and recurrent deletions. A list of genes was generated which scored at the top of each of the three categories

and included nearly all the genes that have been previously implicated in glioblastoma (e.g. IDH1, PIK3C2B, MDM4, MYCN, PIK3CA, PDGFRA, KIT, EGFR, BRAF, PIK3R1, PTEN, RB1, TP53, NF1 and ATRX). The analysis also revealed and validated somatic mutations in 18 new genes. Among the new glioblastoma validated genes were BCOR, and the LRP family member LZTR1 and CTNND2 [20]. BCOR and LRP family member mutations have recently been described in human cancers [21–23]. LZTR1 inactivation drives the self-renewal and growth of glioma spheres in an in vitro culture. CTNND2 inactivation is a key genetic alteration driving the aggressive mesenchymal phenotype of glioblastoma [20].

Altogether, the above observations indicate that important driver mutations and molecular networks have been identified in glioblastoma. However, changes of the gene alterations in time and space, and their relationships with other genetic changes as well as with the tumor's microenvironment remain to be better elucidated in order to develop effective therapies.

B. *Classification of glioblastoma based on expression profiling*

The inability to predict clinical outcomes on the basis of traditional histopathological features illustrated shortcomings in our understanding of glioblastoma biology. Recently, our knowledge concerning the molecular make up of glioblastoma has continuously broadened, and expression profiling revealed that molecular classification of gliomas can be of prognostic value [24, 25]. Philips et al. identified molecular signatures associated with high-grade glioma (HGG) aggressiveness as well as with disease progression and related these signatures to differences in signaling pathways implicated in gliomagenesis [26]. DNA microarray analysis revealed three discrete groups of sample sets that differ markedly in their expression of the survival-related genes. The HGG subclasses were designated proneural, proliferative and mesenchymal based on the gene list that characterizes each subclass. The proneural subtype with intact PTEN and normal EGFR expression was distinguished by markedly better prognosis, and characterized by the expression of genes associated with normal brain tissue and neurogenesis. The two poor prognosis subtypes, characterized by patterns observed in either highly proliferative cell lines or tissues of mesenchymal origin, showed activation of gene expression programs indicative of cell proliferation or angiogenesis [26].

Based on the TCGA genomic profiling, Verhaak et al. identified four subtypes of tumors with a common histological diagnosis of glioblastoma [13]. The subtypes were distinguished by the expression of signature genes and designated according to prior naming: proneural, neural, classical, and mesenchymal. The proneural subtype was associated with younger age, PDGFRA abnormalities, and IDH1 and TP53 mutations. The classical subtype was defined by the constellation of the most common genomic aberrations seen in glioblastoma, with 93 % of samples harboring chromosome 7 amplifications and chromosome 10 deletions, 95 % showing EGFR amplification, and 95 % showing homozygous deletion that spanned the INK4A/ARF locus (CDKN2A). This subclass also showed a distinct lack of additional abnormalities in TP53, NF1, PDGFRA, or IDH1. The mesenchymal subtype was characterized by high expression of CHI3L1 and MET. This subclass showed strong association with a high frequency of NF1 mutations/deletions and low levels of the overall NF1 mRNA expression. The expression patterns of the neural subtype were very similar to those of normal brain tissue specimens [13]. Clinical correlations, however, did not reveal significant survival differences among mRNA-based glioblastoma subclasses [13].

Kong et al. grouped glioblastoma patients into three clusters based on computer-generated nuclear morphometric analyses of large-scale microscopic images [27]. In contrast to the studies by Verhaak et al. [13], this analysis showed significant survival differences and differential therapeutic responses among glioblastoma patients with distinct nuclear morphometry clusters. However, no specific gene mutations correlated with the nuclear morphometry clusters [27].

In another *in silico* analysis of TCGA data, Kim et al. distinguished three groups with different survival characteristics [28]. Median survival of group 3 was markedly longer (127 weeks) than that of groups 1 and 2 (47 and 52 weeks, respectively). This study showed that specific gene expression variations and CNAs have an important role in signaling pathways implicated in gliomagenesis and predicted the clinical outcome of glioblastoma cases. The two poor prognoses subtypes showed more heterogeneity than group 3 with relatively better prognosis. The data also showed that the median survival in a group of patients without codeletion of CDKN2A/2B and PTEN was longer than that in the groups of patients with codeletion of CDKN2A/2B and PTEN. The CNAs in the poor prognosis subgroup were strongly correlated with the epithelial-mesenchymal transition process [28]. This grouping by Kim et al. [28] resembles to the previous molecular clustering of high-grade gliomas by Philips et al. [26].

Doucette et al. analyzed the TCGA glioblastoma data to ascertain the association of antigen expression, immunosuppression, and immune effector response genes within glioblastoma subtypes [29]. The authors defined immune effector gene sets and immunosuppressor genes

by using online knowledge bases (Ingenuity Pathway Analysis, IPA), and a collated list of immune response genes from the literature with an emphasis on those previously documented to have a role in glioblastoma. Analyses of the IPA-selected immune activators and suppressors revealed that the greatest immunologic diversity exists between the proneural and mesenchymal subsets, and that there is a preferential distribution of both proinflammatory and immunosuppressive genes in the latter subset [29].

Altogether, the transcriptome studies greatly complemented data of genomic analyses and facilitated the definition of glioblastoma subclasses with clinical relevance.

C. *Classification of glioblastoma based on epigenome data*

C1 *Gene methylation patterns*

Biological properties of a tumor and its response to treatment are defined not only by chromosomal aberrations and gene expression patterns, but also by epigenetic characteristics such as gene methylation and miRNA-mediated regulations of gene expression. The identification of characteristic methylation patterns has assisted to further refine the molecular classification of glioblastoma. DNA methylation changes are hallmarks of human cancers, in which global DNA hypomethylation is often seen concomitantly with hypermethylation of some CpG islands. Promoter CpG island hypermethylation generally results in transcriptional silencing of the associated gene by blocking the binding of necessary transcription factors to the transcription initiation sites [30]. DNA methylation alterations have been widely reported in human gliomas, and there have been several reports of promoter-associated CpG island hypermethylation in human glioblastoma and other glioma subtypes [11]. Noushmehr et al. determined DNA methylation profiles in a discovery set of 272 TCGA glioblastoma samples and identified a proportion of glioblastoma tumors with highly concordant DNA methylation patterns within a subset of loci, indicative of a CpG island methylator phenotype (G-CIMP) [31]. The G-CIMP-positive samples were found among secondary or recurrent (treated) tumors, and co-occurred with IDH1 mutations. G-CIMP tumors also showed a relative lack of CNAs such as EGFR amplification, chromosome 7 gain and chromosome 10 loss, otherwise commonly observed in glioblastoma. Integration of the DNA methylation data with gene expression data showed that G-CIMP-positive tumors represent a subset of proneural tumors. A comparison of the G-CIMP gene list with prior gene expression analyses suggested that G-CIMP positive tumors may be less aggressive due to the silencing of key mesenchymal genes. Patients with G-CIMP tumors are younger at the time of diagnosis and have relatively better survival times. Thus, the molecular alterations in G-CIMP tumors define a distinct subset of human gliomas with specific clinical features [31]. The age of glioblastoma diagnosis was statistically different between proneural G-CIMP and proneural non- G-CIMP subtypes in a study of Brennan et al. suggesting that the epigenetic features of these transcriptomically similar tumors may reflect distinct etiologies and/or disease characteristics [32].

Li et al. combined the CNA and expression data to provide a more integrated view of the molecular diversity in glioblastoma [33]. The authors took into account the intra-tumoral aneuploidy differences of tumor cells by calculating the aneuploid genome proportion (AGP). This approach revealed a novel glioblastoma subtype (proneural/G-CIMP+) with distinct molecular, clinical, and demographic features. The investigators proposed a hierarchical classification scheme for glioblastoma which integrates diverse molecular and clinical observations. The first step separates proneural/G-CIMP+glioblastomas from non-proneural glioblastomas. Proneural / G-CIMP+samples lacked chromosome 7 gains and chromosome 10 losses that were present in the 3 non-proneural classes. The next step was that many proliferative and classical samples acquired chromosome 13/14/15 deletions and chromosome 19/20 amplifications, respectively, but not both. Mesenchymal samples carried both chromosome 13/14/15 deletions and chromosome 19/20 amplifications but with varying levels of mixing with euploid cells. From these data, the authors proposed a model in which proneural/G-CIMP+glioblastoma may develop in younger patients without chromosome 7 and chromosome 10 CNAs, and IDH1 mutations and/or P53 mutations may be sufficient primary drivers of glioblastoma in these individuals. Patients not carrying IDH1 or P53 mutations in their glioblastomas may acquire chromosome 7 gains and chromosome 10 losses that are accompanied by additional aberrations either in chromosome 13/14/15 in neurons or oligodendrocytes, or in chromosome 19/20 in astrocytes. Depending on the cell lineage, the tumor may evolve into either proliferative or classical subtype. Finally, upon hypoxia, necrosis, and angiogenesis, as well as upon further differentiation, mesenchymal subtypes emerge from these "earlier" classes and carry both chromosome 13/14/15 and chromosome 19/20 abnormalities [33]. While the classification of glioblastoma based on epigenetic observations is very attractive, replications as well as refinements of data are needed.

Although not specific for glioblastoma, the most consistently reproduced and clinically relevant epigenetic biomarker is the promoter methylation status of MGMT (O^6-methylguanine methyltransferase), a DNA repair enzyme that removes alkyl groups from guanine

residues. It is positively associated with glioblastoma sensitivity to alkylating agents, such as temozolomide. The TCGA data raised the possibility that the tumor of patients, who initially responded to the standard frontline therapy, may develop not only treatment resistance, but also a mismatch repair (MMR)-defective hypermutator phenotype. Nevertheless, it has also been recognized that MMR deficiency and MGMT methylation together exert a powerful influence on the overall frequency and pattern of somatic point mutations in glioblastoma in addition to defining treatment response [11].

Of note, Verhaak et al. did not find any association between the glioblastoma molecular subtypes and the methylation status of the MGMT gene [13]. However in the extended TCGA study, the MGMT locus was methylated in 48.5 % of patients, and the G-CIMP cases showed an increased likelihood of having MGMT DNA methylation [32]. When correlated with clinical outcomes, the MGMT status distinguished responders from nonresponders to temozolomide among carriers of classical but not among those of proneural, neural, or mesenchymal tumors. These data provide evidence for MGMT DNA methylation as a predictive biomarker in the classical, but not other subtypes of glioblastoma [32].

C2 *miRNA expression patterns*

miRNAs are a class of short noncoding RNAs, usually 21–24 nucleotides in length that have emerged as key post-transcriptional regulators of gene expression by blocking mRNA translation. Through these mechanisms, miRNA molecules affect important cellular functions such as proliferation, apoptosis, differentiation, and cell signaling. Several of the deregulated miRNAs are cancer-specific and often target key gene products involved in carcinogenesis, invasion and anti-apoptosis of the particular cancer of interest [34]. The TCGA dataset for glioblastoma also includes the expression profiles of miRNAs. Kim et al. used miRNA-expression-based clustering and identified 5 clinically and genetically distinct glioblastoma subclasses, each of which corresponding to a specific neural precursor cell type including "oligoneural", "radial glial", "neural", "neuromesenchymal", and "astrocytic" [35]. The authors observed that miRNA-based consensus clustering yielded robust survival differences among glioblastoma subclasses. The age at diagnosis was also significantly different among subclasses. In addition, a significant survival benefit of radiation and temozolamide was observed for patients with tumors in the astrocytic subclass, but not for those with tumors in the oligoneural, neural, or neuromesenchymal subclasses. Each miRNA-based glioblastoma subclass displayed a distinct pattern of somatic mutations and a unique pattern of CNAs. However, these data did not overlap with the mRNA cluster data [35]. Nevertheless, this study suggests that a miRNA-based classification can assist organizing and maintaining the glioblastoma subclasses. It is important to note, however, that the miRNAs used for classification in this study, were patient survival-related and neurodevelopment-related miRNAs.

In one of the most recent studies, risk scores were calculated to help divide patients into low and high-risk groups [36]. The results revealed that in the five subtypes (proneural-G-CIMP, proneural-non G-CIMP, neural, classical and mesenchymal), patients belonging to the low-risk groups had significantly longer overall survival times than those in the high-risk group. Prognostic miRNA signatures were also identified in the five molecular subtypes. In the classical subtype of glioblastoma, five high risk miRNAs (miR-26a, miR-767-3p, miR-153, miR-31, miR-222), and two protective miRNAs (miR-654 and miR-422b) were found to be significantly correlated with clinical outcomes. In the neural subtype, one high risk miRNAs (miR-222) and seven protective miRNAs (miR-422a, miR-662, miR-566, miR-24, miR-370, miR-492, miR-629) were filtered as signature genes for predicting the patient outcomes. A total of 5 miRNAs (four high risk miRNAs: miR-373, miR-296, miR-191, miR-602; one protective miRNA: miR-223) were selected to identify the overall survival of patients in the mesenchymal subtype of glioblastoma. As for the proneural-G-CIMP and the proneural-non GCIMP subtype of glioblastoma, three (one high risk miRNAs: miR-582 and two protective miRNAs: miR-130a, miR-195) and ten miRNAs (four high risk miRNAs: miR-335, miR-34a, miR-581, miR-21 and six protective miRNAs: miR-361, miR-145, miR-143, miR-378, miR-182, miR-183) were filtered for intrinsic prognostic analysis [36].

Altogether, epigenomic data integrated with previous genomic and transcriptome analyses data led to a refined classification of glioblastoma, which not only better reflects biological properties of the tumor but also better correlate with clinical outcomes including disease prognosis and treatment response.

D. *Heterogeneity of cancer stem cells*

Heterogeneity of glioblastoma not only based on the above discussed DNA aberrations, gene expression changes, or miRNA expression and methylation patterns [37, 38], but also depends on the cancer stem cell profile [39] and the microenvironment. Glioblastomas contain a rare subpopulation of cells with stem cell–like properties, which are called the cancer stem cells (CSC) or tumor-initiating cells. When implanted into nude mice, CSCs give rise to tumors that histologically mimic the original lesions, whereas other cells isolated from the same tumors are non-tumorigenic in vivo [40, 41]. Recent results

indicate that these glioblastoma CSCs also can be divided into subgroups. Lottaz et al. compared the gene expression profiles of 17 glioblastoma CSC lines, and identified two sample clusters [42]. Type I CSC lines highly expressed CD133, PDGFRα, and EGFR, while type II CSC lines expressed CD44 without PDGFRα or CD133. Type II CSC lines also showed EGFR expression. A 24-gene signature reliably differentiated between the two CSC subtypes [42]. Comparing the expression patterns of CSC subtypes with that of high-grade glioma samples published by Phillips and colleagues [26], Lottaz et al. [42] found that the type I CSC subgroup was associated with the proneural phenotype, whereas the type II glioblastoma CSC was associated with mesenhymal phenotype. De Bacco et al. analyzed gene expression profiles of neurospheres isolated and propagated from primary glioblastoma tissues [43]. These neurospheres also could be classified as classical, mesenchymal, or proneural. EGFR protein expression was strongly associated with the classical neurosphere subgroup, whereas MET oncogen protein was associated with the mesenchymal/ proneural neurosphere subgroup. CD133 was inconstantly expressed without association with any subgroup. Interestingly, the neurosphere subgroups expressing MET (MET-pos-NS), showed this marker irrespective of their mesenchymal or proneural profile, while the subgroup lacking MET (MET-neg-NS) displayed significant biologic differences. They had a different proliferation rate, invariably higher in MET-pos-NS. Moreover, the 2 neurosphere subgroups showed a divergent differentiation pattern: MET-pos-NS differentiated along the neuro-astroglial, whereas MET-neg-NS along the neuro-oligodendroglial pathway [43]. Thus, the tumor initiating CSCs also show great heterogeneity and express molecular profiles that define the glioblastoma subtypes. Further studies are needed to better understand their differential roles in tumor development and maintenance.

Altogether, the above genomic, transcriptome and epigenome data represent a major advancement in our understanding of the molecular characteristics and heterogeneity of glioblastoma (Fig. 1). The molecular classification developed in a linear manner built on results evolving from each other, and the integrated analyses of multi-dimensional data provided the most reliable outcome. However, before adapting the classification in clinical practice, more work is needed. As of today, the molecular glioblastoma subgroups are only partially reproducible and the most consistent tumor biomarker remains the MGMT methylation status that predicts the response to temozolomide therapy [44]. The causes of inter-study inconsistencies include the "technical noise" associated with various methods, the biological variability of tumors, the non-normal distribution of data and the non-linear relationship among gene products (gene-expression patterns correlate in a non-linear fashion with biological properties of the tumor) [45]. Further studies are needed to provide a simplified, clinically applicable formula for the characterization of individual tumors including CSCs, which should assist defining prognosis of the disease and guide treatment selection. These efforts likely will have to overcome yet another confounding factor, the intra-tumor heterogeneity of glioblastoma.

Intra-Tumor Heterogeneity of Glioblastoma

It has long been observed that histological features of glioblastoma greatly vary in different areas, and tumor sampling may result in difficulties in establishing even the grade or the cellular origin of a glioma specimen. Consequently, molecular heterogeneity of glioblastoma has also been expected. With the recent developments of molecular technologies, studying

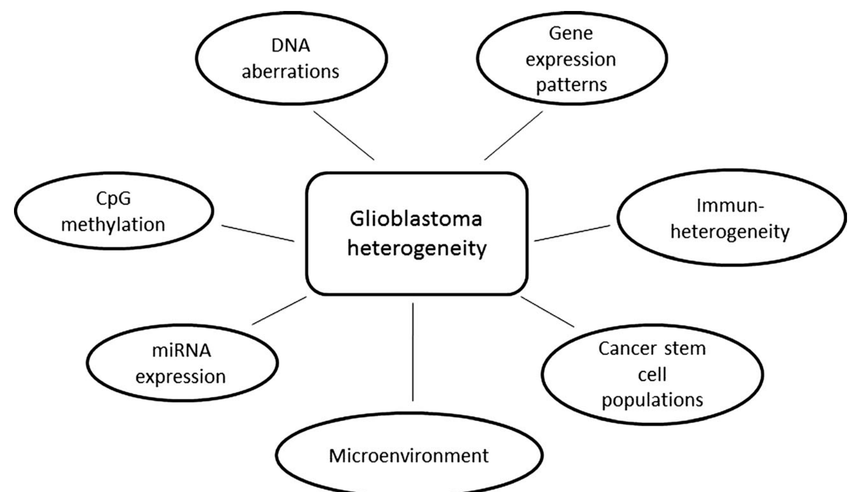

Fig. 1 Factors influencing glioblastoma heterogeneity. This figure depicts in a highly simplified manner those factors that may contribute to defining inter- and intra-tumor heterogeneity of glioblastoma

regional patterns of genomic imbalances within glioblastoma became possible. Nobusawa et al. assessed intra-tumoral genome-wide chromosomal imbalances using a whole genome amplification and CGH approach in two to five small tumor areas on the same histological slide from 14 glioblastomas [46]. The authors found that all glioblastomas had genetic alterations that were common to all areas analyzed and other alterations that were area specific. Genetic alterations that were common to all tumor areas were likely to convey a growth advantage and were considered to represent early genetic events during glioblastoma pathogenesis (driver mutations). These alterations included loss of 10p and 10q, gain at 7p11.2 (EGFR), gain at 1q32.1 (PIK3C2B, MDM4), gain at 4q12 (KIT, PDGFRA), gain at 12q13.3-12q14.1 (CDK4, GLI1), gain at 12q15 (MDM2), loss at 13q12.11–q34 (SPRY2, RB1) and loss at 9p21.1–24.3 (p16INK4a, p14ARF). As indicated, these loci contain well characterized oncogenes and tumor suppressor genes that play important roles in several signaling pathways and that have been reported to be commonly altered in glioblastomas. However, the authors also observed area-specific, functional genetic alterations that may affect the biological behavior of the tumor (e.g. gain at 14q32.33 and amplification of the AKT1). Surprisingly, there were no significant differences in the extent of chromosomal imbalances between tumors with homogeneous and heterogeneous histological features [46].

Snurderl et al. demonstrated coamplification of multiple RTKs in single tumors. These multiple RTK amplifications were not present in the same tumor cell, but were present in distinct intermingled subpopulations of tumor cells [47]. These data were confirmed by Little et al., who found that the two most commonly amplified RTK genes, EGFR and PDGFRA, were present in variable proportions across glioblastomas, with amplification of one or the other gene predominating in certain areas of the same specimen [48]. Moreover, cell lines with different EGFR/PDGFRA profiles derived from the same glioblastoma showed differential responses to growth factors [49].

Sottoriva et al. developed a fluorescence-guided in vivo sampling approach for glioblastoma [50]. During the operation, four to six fragments were obtained from the neoplasms of 11 patients for somatic mutation analyses, while blood samples were also collected from these patients for germline DNA analyses. The authors observed several frequent tumor-specific aberrations that had been reported in other glioblastoma cohorts. Polysomy of chromosome 7 and gain/amplification of EGFR were found in all glioblastomas. Aberrations were present in several other putative glioblastoma driver genes linked to the RAS, p53, and RB pathways. Some of these putative driver aberrations were consistently heterogeneous within the same tumor, including CNAs of the PDGFRA, MDM4, and AKT3 loci, and a deletion of the genomic locus containing PTEN. The CNAs were classified as "common" (all tumor fragments had the CNA), "shared" (more than one but not all fragments had the CNA), and "unique" (only one fragment had the CNA). For every tumor, only a proportion of CNAs were common to all fragments. To characterize intra-tumor variation at the level of transcription, Sottoriva et al. used microarrays and quantified gene expression levels in 51 tumor fragments from 10 patients [50]. Each sample was assigned to one of four subtypes: "proneural", "neural", "classical", and "mesenchymal" using the Verhaak classifier [13]. This study revealed that in 6 of 10 cases, the fragments from the same tumor mass were classified into at least two different glioblastoma subgroups. During the first appearance of a malignant clone, loss of CDKN2A/B and amplification of EGFR, CDK6, and MET were identified. Later malignant events most often included CNAs in genomic regions containing PDGFRA, PTEN, and TP53. By comparing CNA data and measuring mitotic distances among cells of a tissue fragment, the investigators could reconstruct the phylogeny of the cells and the relationships among subclones. The combination of sampling information, reconstructed tumor phylogeny gene expression profiles, and molecular clock data enabled temporal and spatial reconstructions of the tumor ontogeny [50].

Altogether, the cross-sectional studies of molecular aberrations within individual glioblastomas revealed important information about the development of this cancer, and allowed us to gain insights into the likely temporal patterns of mutagenesis defining tumorgenesis. In addition, these studies highlighted the importance of taking intra-tumor heterogeneity into consideration for both diagnostic and therapeutic purposes.

Practical Considerations and Conclusions

The above studies have established that molecular genetic profiles of glioblastoma profoundly influence its biological behaviors and underlie the observed inter- and intra-tumor heterogeneity (Fig. 2). The molecular characteristics also likely define the susceptibility of these tumors to both conventional and targeted therapies, although no study has as yet been published in which patients were treated on the basis of glioblastoma classification, and the real prognostic value of the sub-groups is largely unknown.

Application of the molecular information in the clinic will require new diagnostic capabilities with established sensitivity, specificity and predictive values [51]. It will be important to consider that tumor sampling bias may arise due to differences in somatic events within the primary tumor, between the primary and metastatic sites, among metastatic sites, or even within single biopsies. Nevertheless, it is highly likely that the success of any therapeutic approach will be linked to considerations of glioblastoma heterogeneity.

Fig. 2 Inter- and intra-tumor heterogeneity in glioblastoma. Increasing amounts of data are available concerning inter-tumor heterogeneity and molecular subclasses of glioblastoma. Molecular definition of intra-tumor heterogeneity of glioblastoma is somewhat lagging behind, but the data are rapidly growing

	Inter-tumor heterogeneity	Intra-tumor heterogeneity
DNA aberrations	define different subclasses	show regional variations
Gene expression	defines different subclasses	sorts fragments of the same tumor into different subgroups
- Immun profile	defines immunologic diversity among subclasses	no published data
Epigenome - CpG hypermethylation	defines CpG island methylator phenotype	no published data
- MGMT	is not associated with subtypes but contributes to therapy resistance and hypermutator phenotype	no published data
- miRNA expression	defines different subclasses and can predict clinical outcomes	no published data
Cancer stem cells	defines different subclasses and contributes to immunologic diversity among subclasses	no published data presumably area specific

The above discussed data involve some methodological limitations and translation of the complex multidimensional (genomic, transcriptomic and epigenomic) information in the clinic is not yet straightforward. The complex networks of mutated, over- and underexpressed genes considerably vary among patients and cancer types, reducing the feasibility of creating generally applicable treatment paradigms. The cross-sectional complexity of molecular pathways suggests that a single therapeutic targeting approach may only result in short term success. The surviving tumor cell population may not be a single resistant cancer clone, but rather a heterogeneous population of malignant cells with genetic aberrations that allow them to survive the initial treatment. In addition, a patient-specific dynamics of tumor heterogeneity and evolution also underlie the variations in treatment response.

Nevertheless, it can be predicted with a high probability, that understanding subtype and patient-specific patterns of genetic alterations in tumors will be important for the selection of therapeutic regimens. First, the expression patterns common to a subtype may be informative with respect to the drugs most suitable for a group of patients. For example, the neural glioblastoma subtype has a high rate of EGFR and ERBB2 overexpression, but patients with neural glioblastoma that are not EGFR and/or ERBB2 positive may not benefit from RTK inhibitors. Second, alterations in off-target genes can modulate the efficacy of targeted therapies (i.e., drug resistance). For instance, EGFR-positive non-small cell lung cancers respond to gefitinib, but amplification of the MET proto-oncogene can cause resistance. Breast cancers overexpressing ERBB2 respond to trastuzumab, but PI3K mutation can cause trastuzumab resistance [52, 14]. Third, we have to take into consideration intra-tumor heterogeneity of molecular alterations, and use more innovation in order to successfully treat glioblastoma. Sampling from multiple locations should be required for molecular characterization of the tumor. The molecular diagnosis and therapy selection should go hand in hand. Customized cocktails of drugs may also be necessary to target several different signaling pathways. Alternatively, we need to look for the common signaling "hubs" of the pathways as treatment targets. Fourth, we need to identify main tumor-supportive elements of the microenvironment in order to effectively block cancer growth and spreading. Fifth, new therapies for glioblastoma should not only focus on the inhibition of growth factor signaling, but also consider strategies to differentiate or eliminate CSCs.

Molecular studies of glioblastoma have greatly helped to better understand the pathogenesis of this aggressive tumor [2]. Our knowledge also rapidly broadens regarding glioblastoma initiation and evolution [53]. As a result, first in its history, we are now getting closer to better understand the biology and to succeed with a personalized therapy of glioblastoma.

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