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REVIEW

Molecular and Genomic Alterations in Glioblastoma Multiforme

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In recent years, important advances have been achieved in the understanding of the molecular biology of glioblastoma multiforme (GBM); thus, complex genetic alterations and genomic profiles, which recurrently involve multiple signaling pathways, have been defined, leading to the first molecular/genetic classification of the disease. In this regard, different genetic alterations and genetic pathways appear to distinguish primary (eg, *EGFR* amplification) versus secondary (eg, *IDH1/2* or *TP53* mutation) GBM. Such genetic alterations target distinct combinations of the growth factor receptor—ras signaling pathways, as well as the phosphatidylinositol 3-kinase/phosphatase and tensin homolog/AKT, retinoblastoma/cyclin-dependent kinase (CDK) N2A-$p16^{INK4A}$, and TP53/mouse double minute (MDM) 2/MDM4/CDKN2A-$p14^{ARF}$ pathways, in cells that present features associated with key stages of normal neurogenesis and (normal) central nervous system cell types. This translates into well-defined genomic profiles that have been recently classified by The Cancer Genome Atlas Consortium into four subtypes: classic, mesenchymal, proneural, and neural GBM. Herein, we review the most relevant genetic alterations of primary versus secondary GBM, the specific signaling pathways involved, and the overall genomic profile of this genetically heterogeneous group of malignant tumors. *(Am J Pathol 2015, 185: 1820—1833; http://dx.doi.org/10.1016/j.ajpath.2015.02.023)*

Glioblastoma multiforme (GBM) is a World Health Organization grade IV astrocytoma, which represents the most common and aggressive primary brain tumor. Most GBMs are primary tumors that arise *de novo* as aggressive, highly invasive neoplasias, usually in the absence of clinical, radiological, or histopathological evidence of prior disease and precedent lower-grade lesions; thus, approximately two-thirds of patients with primary GBM have a clinical history of <3 months,[1,2] with rapid development of clinical symptoms. By contrast, secondary GBMs are much less common and they derive from the transformation/progression of lower-grade astrocytomas.[1] Interestingly, these two subtypes of glioblastoma also affect patients at different ages: primary GBM is more common in older patients, whereas secondary GBM tends to occur among patients <45 years. Primary and secondary GBMs are usually indistinguishable on histological grounds, but they show clearly different genetic alterations and genomic profiles (Table 1), supporting the notion that the two groups of GBM arise through different genetic pathways.[23] Herein, we review current knowledge about the signaling pathways most commonly involved in GBM, the molecular and genetic alterations of primary and secondary GBM, including the clinical impact of such alterations, and the most relevant gene expression profiling subgroups of these tumors.

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Table 1 Epigenetic and Genetic Alterations as Well as Gene/Protein Expression Profiles Typically Found in Primary versus Secondary Glioblastomas

Variable	Primary glioblastoma, % (95%)	Secondary glioblastoma, % (5%)	Reference
Promoter methylation			
MGMT	36	75	3
TIMP-3	28	71	4
RB	14	43	5
CDKN2A-p14^{ARF}	6	31	6
CDKN2A-p16^{INK4a}	3	19	6
Genetic alterations			
IDH1 mutation	5	67–85	7,8
IDH2 mutation	0	0	8,9
EGFR amplification	36–60	8	1
TERT mutation	58	28	1,10
CDKN2A-p16^{INK4a} deletion	31–78	19	1
TP53 mutation	28	65	1
PTEN mutation	25	4	1
LOH 10p	47	8	10
LOH 10q	47; 70	54; 63	1,10
LOH 22q	41	82	4
LOH 1p	12	15	11
LOH 13q	12	38	11
LOH 19q	6	54	11
Gene/protein expression profiles			
Fas (APO-1; CD95)*	100	21	12
Survivin*	83	46	13
MMP-9*	69	14	14
EGFR*	63	10	15
EGFR†	High	Low	16
MDM2*	31	0	17
VEGF‡	High	Low	18
VEGF fms-related tyrosine kinase 1§	High	Low	19
IGFBP2§	High	Low	19
Tenascin-X-precursor†	High	Low	16
Enolase 1†	High	Low	16
Centrosome-associated protein 350†	High	Low	16
TP53*	37	97	15
ASCL1¶	33	88	20
Loss of TIMP-3*	17	64	4
PDGFRA/PDGFRB‡	Low	High	20,21
ERCC6†	Low	High	16
DUOX2†	Low	High	16
HNRPA3†	Low	High	16
WNT-11 protein precursor†	Low	High	16
Cadherin-related tumor-suppressor homolog precursor†	Low	High	16
ADAMTS-19†	Low	High	16

Modified from Ohgaki and Kleihues[22] with permission from the American Society for Investigative Pathology.

*Immunohistochemistry.

†Two-dimensional protein gel electrophoresis.

‡Enzyme-linked immunosorbent assay.

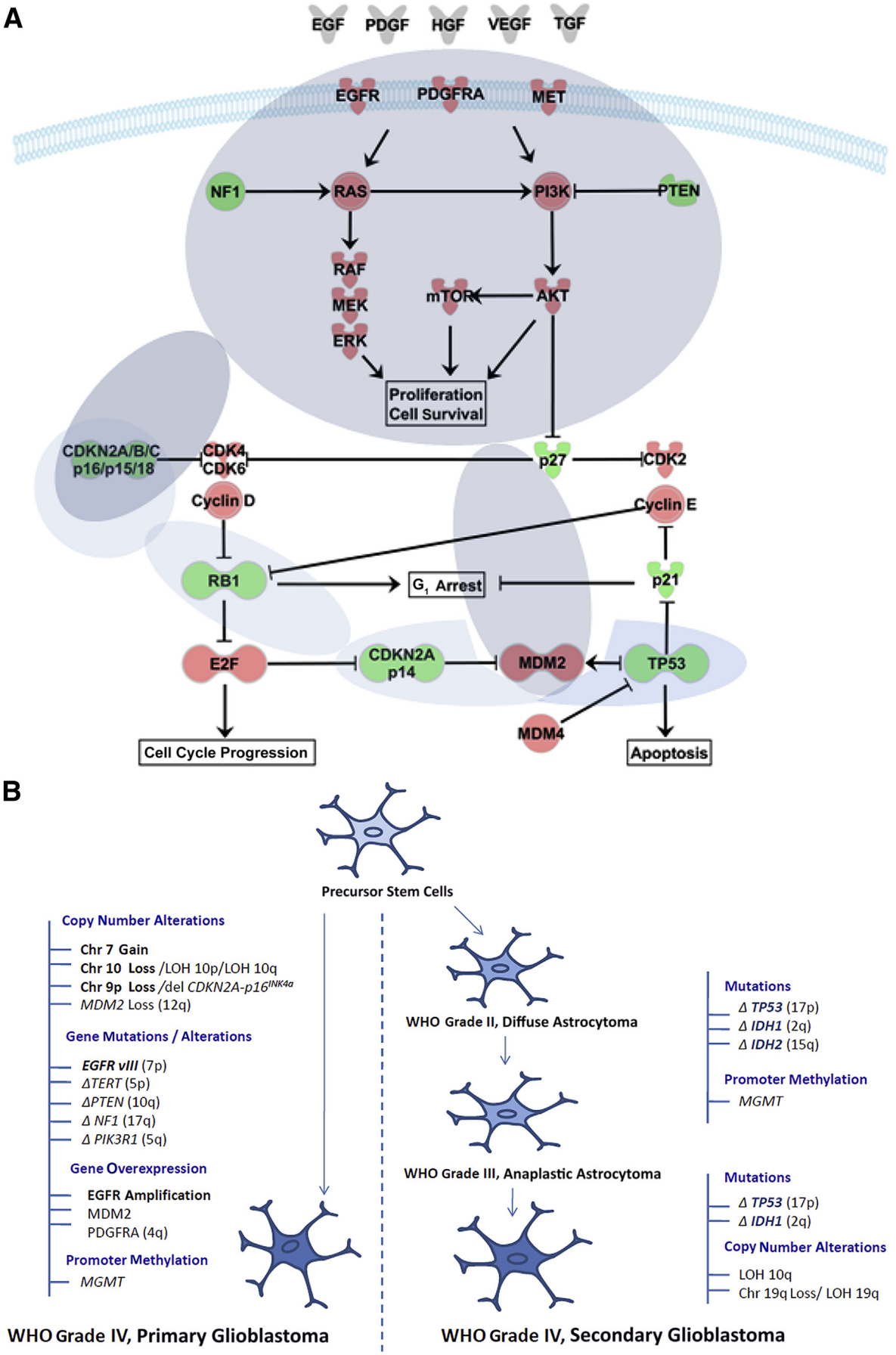
§cDNA array.

¶RT-PCR.

ADAMTS-19, ADAM metallopeptidase with thrombospondin type 1 motif, 19; APO-1, apoptosis-mediating cell membrane protein; ASCL1, achaete-scute complex-like 1; DUOX2, dual oxidase 2; EGFR, epidermal growth factor receptor; ERCC6, excision repair cross-complementation group 6; HNRPA3, heterogeneous nuclear ribonucleoprotein A3; IGFBP, insulin-like growth factor binding protein; LOH, loss of heterozygosity; MDM, mouse double minute; MMP, matrix metalloproteinases; PDGFR, platelet-derived growth factor receptor; TIMP, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor; WNT-11, wingless-type MMTV integration site family, member 11.

Oncogenic Pathways Involved in GBM

The many different genetic and molecular alterations present in GBM lead to modifications of several major signaling pathways that result in brain tumor growth and progression[24,25] (Figure 1A). Although the involvement of several well-known pathways in gliomagenesis is indubitable, there are complex interactions among them, including interactions


A
EGF
PDGF
HGF
VEGF
TGF
EGFR
PDGFRA
MET
NF1
RAS
PI3K
PTEN
RAF
MEK
ERK
mTOR
AKT
Proliferation
Cell Survival
CDKN2A/B/C
p16/p15/18
CDK4
CDK6
p27
CDK2
Cyclin D
Cyclin E
RB1
G1 Arrest
p21
E2F
CDKN2A
p14
MDM2
TP53
MDM4
Cell Cycle Progression
Apoptosis
B
Precursor Stem Cells
Copy Number Alterations
Chr 7 Gain
Chr 10 Loss /LOH 10p/LOH 10q
Chr 9p Loss /del CDKN2A-p16INK4a
MDM2 Loss (12q)
Gene Mutations / Alterations
EGFR vIII (7p)
ΔTERT (5p)
ΔPTEN (10q)
Δ NF1 (17q)
Δ PIK3R1 (5q)
Gene Overexpression
EGFR Amplification
MDM2
PDGFRA (4q)
Promoter Methylation
MGMT
WHO Grade IV, Primary Glioblastoma
WHO Grade II, Diffuse Astrocytoma
Mutations
Δ TP53 (17p)
Δ IDH1 (2q)
Δ IDH2 (15q)
Promoter Methylation
MGMT
WHO Grade III, Anaplastic Astrocytoma
Mutations
Δ TP53 (17p)
Δ IDH1 (2q)
Copy Number Alterations
LOH 10q
Chr 19q Loss/ LOH 19q
WHO Grade IV, Secondary Glioblastoma

with additional unknown players, which potentially contribute to the initiation and transformation of GBM.[26] The most relevant signaling pathways involved in GBM include, among others, growth factor tyrosine kinase receptor (TKR)—triggered pathways, including the Ras sarcoma (Ras) pathway, as well as the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT, retinoblastoma (RB)/cyclin-dependent kinase (*CDK*) *N2A-p16*^{INK4a}, and the TP53/mouse double minute 2 (MDM2)/MDM 4/CDKN2A-p14^{ARF} pathways.

Growth Factor TKR Pathways

Both the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) play an important role in normal and tumoral gliogenesis, through activation of complex intracellular cascades modulated by G-protein—coupled receptors and second messengers that converge at multiple sites. Overexpression of PDGF and EGF receptor (EGFR) in GBM suggests that these TKR-signaling pathways are critical targets in gliomagenesis.[27]

The PDGF family consists of four different ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) that signal through the PDGF receptor (PDGFR) α and PDGFRβ.[28] Both the PDGF ligands and receptors are often co-expressed in glioma cell lines and primary GBM tissues, suggesting the establishment of both autocrine and paracrine signaling loops, which may contribute to tumor formation and progression. PDGFRA and PDGFA are expressed in tumor cells, whereas PDGFB and PDGFRB have been typically found in glioma-associated endothelial cells. Studies on the two new PDGFR ligands PDGFC and PDGFD indicate that they may also play a role in the development of brain tumors. Because co-expression of PDGF and PDGFR has been observed in astrocytomas of all grades, PDGF autocrine signaling may be considered as an early event, with additional secondary alterations in cell signaling being potentially required for progression to GBM. A subset of gliomas characterized by dysregulated PDGFR activity (due to amplification and rearrangement of the *PDGFRA* gene locus and/or overexpression of the PDGF ligand) has been described,[29] and characterized by The Cancer Genome Atlas (TCGA; see below).[21] Interestingly, studies performed on different *in vivo* animal models have shown that PDGFA stimulates the proliferation of PDGFRA-positive neural stem cells residing in the subventricular zone of the adult murine brain, which are able to differentiate and generate glioma-like lesions,[30] suggesting a susceptibility of adult neural stem cells to oncogenic transformation by PDGFRA alteration.

EGFR and its ligands are variably expressed throughout the brain development from embryogenesis into adulthood, suggesting a critical role of EGFR signaling in the proliferation, migration, differentiation, and survival of all types of central nervous system cells and their precursors.[31] In GBM cells, EGFR signaling may be activated in a ligand dependent or independent way, through overexpression of both the ligand(s) and the receptor, leading to an autocrine loop, and via genomic amplification of *EGFR* and/or mutation of the receptor, leading to constitutive activation in the absence of ligand[32]; both types of alterations may coexist in individual GBM. The oncogenic properties of EGFR are associated with a constitutive and uncontrolled increase in its phosphorylation (catalytic) activity. *EGFR* gene mutations/rearrangements and expression of their aberrant protein products are frequently observed in GBM so far; seven common variants have been identified, from which variant 3 (EGFRvIII or del2-7 EGFR, ΔEGFR), which lacks a sequence of 267 amino acids in the extracellular ligand-binding domain leading to a constitutively activated EGFR and pathway, is the most frequent one (it is present in 20% to 50% of GBMs that carry *EGFR* amplification). The introduction of this truncated receptor into glioma cells dramatically enhances their tumorigenicity *in vivo* through both increased cellular proliferation and reduced apoptosis.[33]

Ligand-activated receptors trigger downstream signal transduction pathways, including the Ras/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase (MAPK) pathway, the PI3K/AKT pathway, the protein kinase C pathway, and the STAT pathway, together with vascular endothelial growth factor production, with an impact on cell proliferation, migration, invasion, resistance to apoptosis, and tumor neovascularization.[34]

The Ras Pathway

Ras is a guanosine-nucleotide—binding protein (G-protein), whose activation and deactivation is controlled by cycling

Figure 1 Schematic representation of the different genetic abnormalities and the major signaling pathways involved in the pathogenesis of human glioblastoma. **A:** The interactions between the major signaling pathways altered in primary glioblastoma multiforme (GBM; dark gray) and secondary GBM (light gray). Genes that are inactivated or hyperactivated by different mechanisms are shown in green and red, respectively. In the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt signaling pathway, the growth factor receptor becomes activated and recruits PI3K to the cell membrane, converting phosphatidylinositol-4,5-bisphosphate to the PIP3 second messenger molecule. Downstream effector molecules, such as AKT and the mammalian target of rapamycin (mTOR), are then activated, which help to induce cell proliferation and block apoptosis. PTEN terminates the phosphatidylinositol 3,4,5-trisphosphate (PIP3) signal; inactivation leads to increased availability of PI3K, and PI3K activates AKT, which, in turn, leads to an increased proliferative activity and survival. In the TP53 pathway, *TP53* mutation or increased degradation of TP53 because of increased mouse double minute (MDM) 2 activity interrupts the normal cell cycle arrest and apoptosis after DNA damage. In the retinoblastoma protein (RB) pathway, cyclin-dependent kinase (CDK) 4 and CDK6 form complexes with members of the cyclin D family and phosphorylate the RB, which releases the E2F transcription factor, inducing cell proliferation by transcription of genes that promote DNA synthesis. Loss of RB leads to elevated levels of E2F and, therefore, cell proliferation. **B:** The most frequent and relevant molecular abnormalities of primary versus secondary GBM are shown. Chr, chromosome; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal—regulated kinase; HGF, hepatocyte growth factor; LOH, loss of heterozygosity; NF1, neurofibromatosis type I; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; WHO, World Health Organization.

between the active GTP-bound and inactive GDP-bound forms. Receptor-mediated activation of Ras and Ras activation by oncogenic mutations are common in human tumors and contribute to the development and maintenance of the malignant phenotype. For example, the active Ras-GTP protein, through its downstream effectors, including the Raf, PI3K, and Ral-guanine nucleotide exchange factors, promotes cell cycle progression, survival, and migration.[35]

An increased Ras pathway activity is observed in virtually all GBMs.[36] Interestingly, *RAS* mutations are rarely found in GBM. However, high levels of Ras-GTP have been documented in GBM cell lines and primary tumors, suggesting that this signaling pathway is activated by upstream factors, such as TKR activation (eg, EGFR or PDGFR), which might be required for tumor induction, progression, and maintenance. Ras-GTP is downstream of growth factor receptors at a major signal transduction crossroad, translating extrinsic messages into the Raf-kinase/MAPK/extracellular signal–regulated kinase pathway, PI3K/AKT, or PI3K/Rac/Rho pathways, influencing cell proliferation, survival, and migration. In fact, MAPK/extracellular signal–regulated kinase signaling, one of the downstream effectors of Ras, is also increased in human GBM.[34] In GBM, the existence of mutations affecting the expression or the activity of MAPKs has not been reported. However, because MAPKs may contribute to cell proliferation and could be activated in response to the inhibition of PI3K/mammalian target of rapamycin, their role in gliomagenesis is currently under investigation. In addition, the Ras signaling pathway may also be activated through the loss of function of neurofibromatosis type I (NF1), a negative regulator of Ras. Germline and loss-of-function *NF1* mutations have been identified in neurofibromatosis type I, which includes GBM as part of the clinical spectrum. In this regard, the results of TCGA Consortium showed that approximately 20% of GBMs harbor loss-of-function mutations of the *NF1* gene.[24]

The PI3K/PTEN/AKT Pathway

Elevated signaling through the PI3K-mediated cell signaling pathway has been implicated in the pathogenesis of GBM. Recruitment of PI3K to the cell membrane activates downstream effector molecules, such as AKT and mammalian target of rapamycin, resulting in cell proliferation and increased cell survival by blocking apoptosis.[37] The *PTEN* tumor-suppressor gene negatively regulates PI3K, but its function is frequently lost in GBM because of loss of heterozygosity (LOH) at the 10q23.3 locus or because of gene mutations (15% to 40%),[38] causing constitutive activation of the PI3K pathway and higher levels of activated AKT in glioma cells. Some reports also indicate that PTEN plays a significant role in inducing G_1 cell cycle arrest and apoptosis, along with regulation of cell differentiation. Tumors with an activated PTEN/AKT pathway may be sensitive to mammalian target of rapamycin inhibitors (eg, rapamycin).[39] Several studies using viral vectors have shown that wild-type PTEN suppresses tumorigenicity of glioma cells and inhibits the PI3K/AKT pathway.[40] Thus, the PI3K/PTEN/AKT pathway appears to play a role in gliomagenesis, as also supported by the findings of the pilot project of the TCGA, which identified alterations in the EGFR/Ras/NF1/PTEN/PI3K pathway in 88% of all GBM patients.[24]

The RB/CDKN2A-p16^{INK4a} Pathway

This pathway plays a central role in the regulation of cell cycle and cell proliferation, because its components are activated and/or inhibited by growth-promoting as well as growth-suppressing signals.[41] The RB gene (13q14) encodes the RB phosphoprotein; in quiescent cells, RB is in a hypophosphorylated state (active) bound to E2F, preventing transcription of genes important for mitosis and, thus, preventing progression through the G_1/S cell cycle checkpoint. In proliferating cells, growth factors lead to the induction of cyclin D1, as well as to the activation of CDK2/cyclin E through degradation of its inhibitor, p27^{Kip1}. These activated CDK-cyclin complexes trigger phosphorylation of RB in late G_1 phase, which is maintained later in the S, G_2, and M phases. Phosphorylated (inactive) RB enables E2F release, leading to transcriptional activation of growth-promoting genes, required for DNA synthesis and cell growth.[37] Negative regulators of the RB signaling pathway include the Ink4 family of proteins (CDKN2A-p16^{INK4a}, CDKN2B-p15^{INK4b}, CDKN2C-p18^{INK4c}, and CDKN2D-p19^{INK4d}) that compete with the D-cyclins for CDK4/6, to prevent the formation of the active kinase complex that phosphorylates RB.[41]

Inactivation of the RB pathway through disrupted RB-E2F interaction may occur by mutation of the *RB* gene itself, by loss of RB expression, or by the inactivation of RB by phosphorylation through CDK/cyclin complexes. CDKs can be activated by an increase in the amount of the catalytic enzyme, by an increase in the amount of their cofactors (cyclins), and/or by a decrease in the amount of endogenous CDK inhibitors (eg, CDKN2A-p16^{INK4A}). In addition, the CDKN2A genetic locus at chromosome 9p21 produces both CDKN2A-p14^{ARF} and CDKN2A-p16^{INK4a} by alternative splicing.[42] Because p16^{INK4a} negatively regulates CDK4 and p14^{ARF} inhibits MDM2, leading to a rapid blockade of the ubiquitin-mediated decay of TP53, simultaneous inactivation of both genes by homozygous deletion dysregulates both the RB and the TP53 pathways.[43]

The RB pathway is altered in 78% of 206 primary GBMs,[34] either directly by mutations, deletions, or promoter methylation at the *RB* locus or indirectly through alterations on the RB positive and negative regulators. In turn, *RB* promoter methylation and gene silencing are more frequently found in secondary (43%) than in primary (14%) GBMs.[44] Alterations in the RB pathway include homozygous deletion and mutation of *CDKN2A-p16^{INK4a}* and *RB* in 52% and 11% of the samples, respectively, and homozygous deletion of *CDKN2B-p15^{INK4b}* and *CDKN2C-p18^{INK4c}* in 47% and 2% of the tumors,

respectively. In turn, *CDK4*, *CDK6*, and *CCND2* (cyclin D2) gene amplification has been found in 18%, 1%, and 2% of GBMs, respectively.[24] Activated AKT deregulates cell growth by stabilization of cyclin D and promotion of nuclear entry of MDM2, leading to degradation of TP53. AKT might also inhibit p21 expression through its phosphorylation and activation of MDM2. In addition, activated AKT exerts an anti-apoptotic activity by phosphorylating and inactivating pro-apoptotic signaling proteins (eg, BAD and caspase 9), and it may contribute to tumor invasion and metastasis by stimulating secretion of matrix metalloproteinases.[45]

The TP53/MDM2/MDM4/CDKN2A-p14^{ARF} Pathway

The TP53 protein, coded by the *TP53* gene at chromosome 17p13.1, plays a role in the cell cycle, cellular responses to DNA damage, cell death, and differentiation. It is a sequence-specific nuclear transcription factor that binds as a tetramer to defined consensus sites within the DNA, affecting the transcription of its target genes, by either transcriptional activation or modulation of the activation of other proteins through direct binding.[43] The TP53 network is activated in response to cellular stress conditions and facilitates DNA repair or induces cell death in case of too much damage, preventing cells with mutated or damaged DNA from dividing. TP53 transcriptionally regulates the promoters of potential effector genes, such as p21, which blocks cell cycle progression at the G_1 phase by binding and inhibiting the function of cyclin-D proteins.[46] This gives time for DNA repair before replication or mitosis and, thereby, links p21 directly to the tumor-suppressor function of TP53. Moreover, the TP53 and the RB pathways interact with each other via p21.

Recent findings show that TP53 also regulates proliferation, differentiation, and survival of stem cells, further highlighting the relevance of TP53 in suppressing GBM.[47] After stress, the activity of TP53 is blocked by its negative regulator MDM2, whose transcription is induced by TP53, generating a negative feedback loop that regulates the activity of TP53 and the expression of MDM2.[43] In addition, activation of TP53 is achieved through inactivation of MDM2 by CDKN2A-p14^{ARF} binding. The MDM4 TP53 binding protein homolog (also called MDMX) also regulates TP53 activity, and p14^{ARF} is negatively regulated by TP53.

The TP53 signaling pathway is disrupted in GBM due to *TP53* mutation and/or amplification, overexpression of MDM2, and/or loss of expression of CDKN2A-p14^{ARF}, all such alterations blocking TP53 activity and potentially leading to uncontrolled cell proliferation and tumor formation. In human gliomas, *TP53* mutations are missense mutations and target the highly conserved domains of *TP53* in exons 5, 7, and 8, which are crucial for DNA binding. At least one alteration in the TP53/MDM2/CDKN2A-p14^{ARF} pathway has been reported to occur in approximately 50% of primary glioblastomas and in >70% of secondary glioblastomas.[48] Consistently, the TCGA pilot project showed an overall frequency of genetic alterations in the TP53/MDM2/MDM4/CDKN2A-p14^{ARF} pathway in glioblastomas of 87%, with such alterations being associated with *TP53* mutation or homozygous deletion in 35% of cases, *MDM2* amplification in 14%, *MDM4* amplification in 7%, and CDKN2A-*p14^{ARF}* homozygous deletion or mutation in 49% of all GBM cases.[24] Amplification of *MDM4* was detected in 4% of GBM with neither *TP53* mutation nor *MDM2* amplification.[49]

Epigenetic Changes in GBM

Gliomas present a variety of epigenetic alterations that induce changes in normal gene expression, without altering the DNA sequence. Aberrant epigenetic mechanisms, such as DNA methylation, histone modifications, chromatin remodeling, or altered noncoding RNA expression (eg, miRNAs), are currently recognized as relevant events in tumor formation, in addition to classic genetic alterations.[50] Until now, most studies about the epigenetic changes of glioblastoma have focused on DNA methylation, including hypermethylation of CpG islands (associated with tumor-suppressor gene silencing), gene-specific hypomethylation (resulting in aberrant activation of oncogenes), and genome-wide hypomethylation (potentially leading to chromosomal instability, loss of imprinting, and uncontrolled cell proliferation).[51] To date, multiple changes in the DNA methylation pattern of promoters of genes involved in cell cycle regulation (eg, *CDKN2A-p16^{INK4a}* and *CDKN2B-p15^{INK4b}*), tumor suppression (eg, *RB*, *VHL*, *EMP3*, *RASSF1A*, and *BLU*), DNA repair, and genome integrity (eg, *MGMT* and *hMLH1*), as well as genes associated with regulation of tumor invasion and inhibition of apoptosis (eg, *DAPK1*, *TIMP3*, *CDH1*, *PCDHGA11*, and *TMS1/ASC*),[5,52–55] have been reported in GBM.

Genetic Alterations of Primary versus Secondary GBM

Overall, primary GBMs typically harbor three predominant genetic alterations, as confirmed also by high-density single-nucleotide polymorphism arrays[56]: amplification and/or a high rate of *EGFR* mutation in chromosome 7p, homozygous deletion of the *CDKN2A-p16^{INK4a}* gene in chromosome 9p (with absence of the CDKN2A-p16^{INK4a} α and/or the CDKN2A-p14^{ARF} β transcripts), and deletion of the *PTEN* gene typically in association with monosomy 10.[56–58] In addition, amplification of the *MDM2* oncogene is also present in a smaller, but significant, percentage of primary GBMs (<15%), particularly among those primary glioblastomas that lack *TP53* mutations and telomerase reverse transcriptase (*TERT*) promoter mutations[59,60] (Figure 1B).

Amplification and/or mutation of the *EGFR* gene (7p11.2) occur in 36% to 60% of primary GBMs. The most common EGFR mutant type, variant 3 (EGFRvIII), is due to

an 801-bp in-frame deletion of exons 2 to 7, and it leads to a constitutively active protein, resulting in increased proliferation and survival of mutated cells.[33] In most GBM tumors, overexpression of the EGFRvIII mutant coexists with *EGFR* gene amplification, but overexpression of the EGFRvIII mutant without *EGFR* amplification has also been reported in a small proportion of primary GBMs.[61] All primary glioblastomas with *EGFR* amplification show EGFR overexpression, and 70% to 90% of cases with EGFR overexpression have *EGFR* amplification.

Homozygous deletion of *CDKN2A-p16*^{INK4a} is also more frequently observed among primary than secondary glioblastomas, whereas other alterations of this gene predominate in the latter tumors.[6] Chromosome 9p deletions involving the *CDKN2A-p16*^{INK4a} gene typically target other closely located genes, such as the methylthioadenosine phosphorylase (*MTAP*) gene, whose relevance in GBM remains to be established.[62]

LOH of chromosome 10 is present in up to 70% of primary glioblastomas,[1,63] suggesting the presence of several tumor-suppressor genes in this chromosome, which may be involved in GBM tumorigenesis. Three main chromosomal regions are commonly deleted: 10p14-15, 10q23-24 (*PTEN*), and 10q25-pter. Interestingly, *PTEN* gene mutations have been reported in approximately 25% of all glioblastomas,[22] with such mutations being almost exclusively detected among primary glioblastoma[1]; by contrast, *PTEN* homozygous deletion occurs rarely. More than half of *PTEN* mutations result in the introduction of premature stop codons, leading to translation of truncated proteins. Alterations involving the *PTEN* gene rarely occur in low-grade gliomas, whereas the frequency of inactivation of the *PTEN* gene increases in the more advanced stages of the disease (Figure 1B). Interestingly, primary GBMs frequently show loss of chromosome 10 in association with *EGFR* amplification, raising the possibility that the interaction between EGFR signaling and functional abrogation of relevant chromosome 10 suppressor genes may contribute to the aggressive features of GBM.

On the basis of the pilot project of the TCGA Consortium and other studies, additional genetic alterations have been identified in primary GBM, which include *NF1* mutation/homozygous deletion (18%) and *PIK3R1* (regulatory subunit 1 of phosphatidylinositol 3-kinase) mutations (10%).[24] In turn, mutation and/or amplification of the *PIK3CA* gene, which codes for the catalytic subunit α of the phosphatidylinositol-4,5-bisphosphate 3-kinase, is a rare event in both primary and secondary GBMs (approximately 5% and approximately 13%, respectively).[64]

In contrast to primary GBM, *TP53* mutations at chromosome 17p are most frequently seen in secondary GBM, mainly involving codons 248 and 273 and G:C → A:T mutations at CpG (cytosine-phospho-guanine) sites. *TP53* mutations in secondary GBM are already detectable at the early stages of the disease,[1] and they seem to be an early event associated with malignant transformation in the pathway to secondary GBM (Figure 1B). Interestingly, methylation of the promoter of the *MGMT* DNA repair gene is also frequently associated with G:C → A:T mutations of the *TP53* gene.[3,65] In addition, isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* mutations have been identified recently, as early genetic alterations present in most low-grade gliomas, as well as in the pathway to secondary glioblastomas. However, they are rare among primary GBMs.[7] In gliomas, *IDH1/2* mutations are associated with an increased DNA hypermethylation profile[66]; therefore, it is likely that *IDH1/2* mutations are involved in oncogenesis through inactivation of tumor-suppressor genes via hypermethylation of their promoters, which is facilitated by the mutated gene/protein. Gliomas with mutated *IDH1* and *IDH2* have a better prognosis compared with gliomas with wild-type *IDH*.[8] Inactivation of the proapoptotic Harakiri (*HRK*) gene, which encodes for a bcl2-interacting protein at 12q24,[67] because of hypermethylation of its promoter, could play a critical role in the development and progression of secondary GBM via abrogation of apoptosis in tumors expressing wild-type TP53.

Partial LOH of chromosome 10q is also a frequent event in secondary glioblastomas.[10] LOHs of chromosome 13q (typically including the *RB* locus), chromosome 19q (with a common deleted region at 19q13.3),[11] and chromosome 22q (including the *TIMP-3* putative tumor-suppressor gene at 22q12.3)[4] have been detected more frequently in secondary versus primary GBM. Less commonly than in primary GBMs, secondary GBMs also display amplification of the *PDGFRA* and/or *PDGFRB* genes, potentially in association with overexpression of the PDGF ligand, yielding to an autocrine loop in GBM tumor cells. Table 1 summarizes all of the above described and other genetic alterations and altered gene/protein expression profiles associated with primary versus secondary GBM.[12–20]

Genetic and Molecular Alterations of GBM with Clinical Impact

In the past decades, several alterations reported in GBM have emerged as being of potential clinical relevance for more accurate diagnostic classification, prognostic stratification, and/or prediction of response to therapy in GBM. Among others, amplification of the *EGFR* gene, *IDH1/IDH2* mutations, and hypermethylation of the promoter of the O(6)-methylguanine-DNA methyltransferase (*MGMT*) gene have become particularly relevant from the clinical point of view, in addition to detection of codeletion of chromosomes 1p and 19q in low-grade gliomas. In turn, gene expression profiles (GEPs) are still not yet being generally used.

EGFR Amplification and EGFR Genetic Variants

EGFR is a transmembrane glycoprotein that acts as a TKR. Once bound to its ligand, *EGFR* becomes autophosphorylated and induces subsequent activation of signal transduction pathways involved in the regulation of cell proliferation, differentiation, and survival. Although present in normal

cells, the *EGFR* gene is the most frequently overexpressed gene, mainly in primary GBM,[1,22] and its alterations in gliomas are highly associated with high-grade malignancy.[68] However, controversial results exist regarding the prognostic significance of *EGFR* amplification in GBM. Although some studies reported that *EGFR* amplification is associated with a poor prognosis and a shorter survival of GBM patients,[69] others claim that such association with survival would not be significant or that it could even be associated with a better outcome.[70] In turn, some reports have found a poor prognosis for GBM patients carrying amplification of the *EGFR* gene among all age groups, whereas others have found *EGFR* amplification to be a predictor for prolonged survival only among older patients. Such discrepant results could potentially be associated with the specific underlying genetic lesion targeting the *EGFR* gene. In this regard, the prognostic impact of *EGFRvIII* (the most common *EGFR* mutant variant, which leads to constitutive activation of EGFR in a ligand-independent way) has not been investigated as extensively as *EGFR* amplification. Despite this, controversial results have also emerged. Accordingly, the EGFRvIII variant has been found not to be related to patient outcome,[71] to be associated with an unfavorable prognosis,[72] or even to be a molecular predictor for prolonged overall survival among (conventionally) treated GBM patients.[73] Some other polymorphic genetic variants of the *EGFR* gene (eg, the -191C/A polymorphism involving the promoter region of the gene) have also been associated with the prognosis of glioma patients,[74] but its clinical value still deserves confirmation.

In addition, it has also been speculated that the EGFR status could contribute to predict response to EGFR-targeted therapies. In fact, several mono and multiple EGFR-targeted therapies have been developed and assayed in recent years, including antibodies and small-molecule inhibitors of EGFR. Among other agents, two small-molecule EGFR inhibitors (gefitinib and erlotinib) have been extensively tested, but the response to these drugs (eg, erlotinib as a single agent) did not seem to be effective.[75] Other EGFR-directed therapies, such us RNA-based treatment approaches, ligand-toxin conjugates, and radioimmunoconjugates, are currently at various stages of evaluation for clinical purposes.[33,76] In parallel, efforts are being made to increase the knowledge about the mechanisms underlying resistance to EGFR-targeted therapies and to clarify the clinical value of *EGFR* amplification and its variants (eg, EGFRvIII) for prognostic stratification of GBM.[61]

IDH1/IDH2 Mutations

IDHs catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate and reduce NAD^+ and $NADP^+$ to NADH and NADPH, respectively.[77] IDH1 is localized in the cytoplasm and the peroxisomes, whereas IDH2 is in the mitochondria,[78] where they are involved in the tricarboxylic acid cycle, as well as in protection against oxidative stress. IDH1 participates in the lipid and glucose metabolism.[79]

Parsons et al[80] revealed, by gene sequencing of 22 GBMs, the presence of recurrent (12%) point mutations in the active site of *IDH1*, a gene that had never been linked to cancer before. Furthermore, it was found that other GBMs, which do not have *IDH1* mutations, may show mutation of the *IDH2* gene.[8] *IDH1* (coded at chromosome 2q33) and *IDH2* (coded at chromosome 15q26) mutations typically consist of monoallelic, somatic, and missense changes. Mutations of *IDH1* almost always affect the *R132* codon,[80] and *IDH2* mutations exclusively affect the *R172* and *R140* codons.[8] Both *IDH1* and *IDH2* mutations are more frequently detected among grade II to III gliomas and secondary glioblastoma (70% to 75%), whereas they are rare in primary glioblastoma (5%).[7,81] Interestingly, the occurrence of *IDH1* mutations in diffuse gliomas is strongly associated with *TP53* mutation and also del(1p)/del(19q), indicating that they may represent an early event.[82] Both younger age and mutated *TP53* have been reported to be associated with a better outcome among GBM patients. Noteworthy, *IDH1* mutations are present in a higher fraction of younger patients, who also carry a high frequency of *TP53* mutations, all being typical features of secondary GBM.[9,83] Other genetic associations that have been reported in gliomas include *IDH1* mutation and *MGMT* promoter methylation, in the absence of chromosome 10 losses and *EGFR* amplification.[84] Altogether, these associations might contribute to explain why *IDH1/IDH2* mutations have been found to be positive prognostic factors.[9,84]

Efforts are ongoing to better understand the role of *IDH1* mutations in gliomagenesis. Initially, it was postulated that mutations could abrogate the function of the protein, leading to reduced synthesis of the α-ketoglutarate metabolite,[85] but striking new pieces of evidence have shown that IDH1 mutants also confer an enzymatic gain-of-function phenotype, associated with production of the alternative metabolite, 2-hydroxyglutarate; because 2-hydroxyglutarate may contribute to gliomagenesis,[86] a new hypothesis has been raised about whether treatments that would reduce production of 2-hydroxyglutarate could be effective against gliomas with *IDH1* mutation.

Hypermethylation of the MGMT Promoter

The *MGMT* gene (coded at chromosome 10q26) is frequently silenced in GBM by promoter hypermethylation in association or not with monosomy 10/del(10q). Currently, this represents one of the most relevant prognostic factors in GBM and a potent predictor of response to treatment with alkylating agents.[87] Accordingly, an association between methylation of the *MGMT* gene promoter and response to alkylating chemotherapy using nitrosourea compounds, temozolomide (TMZ), or a combination of both[88] has been observed in GBM. Several alkylation sites have been described in the DNA, as targets for these cytotoxic compounds, the most frequent site being the O6-position of guanine. The use of alkylating chemotherapeutic drugs, such as TMZ, causes binding of an alkyl group to the O6-position

of guanine, thereby inducing a DNA mismatch, DNA double-strand breakage, and ultimately apoptosis of proliferating cells. Thus, the MGMT protein counteracts the normally lethal effect of TMZ by repairing DNA damage. When a tumor has a hypermethylated *MGMT* promoter, transcription of the gene is blocked, leading to lack of MGMT mRNA and protein expression, while enhancing the cytotoxic effects of the alkylating drug(s).

Previous studies have shown that patients with hypermethylation of the *MGMT* gene promoter may have longer survival rates when treated with both TMZ and radiotherapy (median survival at 2 and 5 years of 49% and 14%, respectively) instead of radiotherapy alone (median survival at 2 and 5 years of 24% and 5%, respectively).[83,89,90] However, it has been recently reported that methylation of the *MGMT* promoter could also be predictive of response to radiotherapy and a longer survival in GBM, in the absence of adjuvant chemotherapy.[91] Therefore, it remains unclear whether MGMT also plays a role in repairing radiotherapy-induced DNA damage, whether other DNA repair genes than *MGMT* are also silenced by promoter hypermethylation, or whether the survival advantage of such *MGMT* hypermethylated cases is better explained by other coexisting prognostically favorable genetic features frequently observed in low-grade oligodendrogliomas and secondary GBM, such as codeletion of chromosomes 1p and 19q[92] and *IDH1* mutation.[84]

Despite its recognition as a favorable prognostic marker, *MGMT* testing is still not widely used in the day-to-day laboratory routine of most centers; this is mainly due to the lack of technical standardization[65,93] because of sample heterogeneity and lack of precise identification/validation of specific *MGMT* promoter regions predictive of patient response to therapy. In addition, several studies indicate that the pattern of expression of the MGMT protein is associated with neither the methylation status of the *MGMT* promoter nor the patient outcome, suggesting that other molecular mechanisms, in addition to *MGMT* promoter methylation and del(10q)/monosomy 10, may be involved.[94]

GEP of GBM and Its Molecular/Genomic Subtypes

In the past decade, efforts have been made to further define the impact of genetic/molecular alterations of GBM at the genomic level. Recent availability of high-throughput microarray-based assays allowed for genome-wide quantitative analysis of thousands of genes simultaneously, providing new insights into patterns of overexpressed and underexpressed genes potentially involved in malignant transformation and disease progression. Early GEP studies identified differentially expressed genes among histopathologically defined subtypes of gliomas (eg, GBM versus oligodendrogliomas,[95] low- versus high-grade astrocytomas,[95] primary versus secondary GBM,[96] or even GBM with versus without *EGFR* amplification).[95] Altogether, these results indicate that histopathological subtypes of gliomas, and also genetic subgroups of GBM, show unique GEP at the mRNA transcriptional level that may potentially be used to distinguish among them.

Despite this, discordant results between morphological and molecular tumor subtypes, as defined by GEP, have been also frequently observed among histologically ambiguous gliomas. In this regard, the potential diagnostic utility of GEP for the molecular classification of gliomas has been highlighted by Nutt et al,[97] who identified two sets of genes significantly associated with classic GBM or anaplastic oligodendroglioma, and built a class prediction model that showed an accuracy of 86% in assigning diagnostically challenging samples. In another collaborative study, Parsons et al[80] discovered a variety of genes that, at that time, were unknown to be altered in GBM (eg, recurrent mutations of the *IDH1* gene were detected in 12% of the cases).

In addition, several GEP studies have identified distinct molecular subtypes of gliomas with prognostic significance. Early studies by Freije et al[98] identified 595 differentially expressed genes that correlated with overall survival in 74 gliomas. Such molecular subtypes [hierarchical clustering (HC) 1A, HC1B, HC2A, and HC2B] could be further segregated into two groups of patients with distinct survival (median overall survival of 4.8 and 0.6 years) through HC. Nutt et al[97] showed that the prognostic impact of the survival cluster on the basis of tumor GEP was independent of patient age and histological grade. Interestingly, this study also showed that one subtype was enriched for genes involved in neurogenesis, whereas the poor survival subtypes were enriched for genes associated with cell proliferation and extracellular matrix/invasion, suggesting a more versus less differentiated phenotype for the two groups, respectively.

Later, Phillips et al[99] subdivided GBM into three groups [eg, mesenchymal (MQ), proliferative (PF), and proneural (PN)], according to the similarities observed between distinct tumoral GEP and both key stages in neurogenesis and known cell types. MQ, PF, and PN signatures were associated with those of neural stem cells, transit-amplifying cells, and immature neurons, respectively. The PN signature is typically overrepresented among less aggressive forms of high-grade gliomas, whereas the MQ and PF subtypes were enriched in more aggressive high-grade tumors; in addition, such classification also showed an association with clinical outcome. A subsequent meta-analysis on 267 GBMs, on the basis of previously published data together with new GEP data,[100] identified 377 differentially expressed genes, which classified GBM into four distinct subtypes by HC analyses: HC1A/PN, HC2A/PF, HC2B/MQ, and a fourth subtype with HC2A/HC2B hybrid features, termed PF/MQ. Survival analysis confirmed the more favorable outcome of HC1A/PN GBM versus the other three subtypes. Moreover, younger GBM patients more frequently had PN GBM type tumors, which could contribute to explain their longer life expectancy and their better outcome.

Vital et al[95] investigated the association between the GEP and both the cytogenetics and histopathology of 40 gliomas.

Table 2 Classifications of GBM Proposed on the Basis of Different Profiles of Molecular and Genetic Alterations Identified and the Relationship Existing between the Different Subtypes Identified

	GBM classifications		
Reference	Philips et al[99]	Verhaak et al[21]	Crespo et al[56]
Samples analyzed, *N*	76	170	35
Molecular/genetic subgroups of GBM	Proneural	Proneural	Pattern III
	Normal *EGFR*	*PDGFRA* AMP	+7
	Normal *PTEN*	*IDH1* MUT	del(9p21)/+9q
		TP53 loss/MUT	Normal 10
		PI3KCA/PIK3R1 MUT	
	Proliferative and mesenchymal	Classical	Pattern I
	EGFR AMP/normal	*EGFR* AMP	*EGFR* AMP
	+7 and −10	Loss 10 (*PTEN*)	−10/del(10p) and del (10q)
	PTEN loss	Loss 9p21 (*CDKN2A*)	del(9p21)
		Mesenchymal	Pattern II
		Loss 17q11.2 (NF1)	+7
			del(9p21)
			−10/del(10p) and del (10q)
		Neural	Pattern IV
		Not distinctive*	+7
			Normal 9p21
			−10
			Pattern V
			+7
			+9
			−10

*Expression of neuron histological markers.

AMP, gene amplification; GBM, glioblastoma multiforme; MUT, gene mutation; NF1, neurofibromatosis type I.

Unsupervised and supervised analyses showed significantly different GEP in low- versus high-grade gliomas, the most discriminating genes including genes involved in the regulation of cell proliferation, apoptosis, DNA repair, and signal transduction. In turn, among GBM, three subgroups of tumors were identified according to their GEP, which were closely associated with the cytogenetic profile of their ancestral tumor cell clones: i) *EGFR* amplification, ii) isolated trisomy 7, and iii) more complex karyotypes. In this regard, we have also investigated the direct impact of cytogenetic alterations defined by copy number changes on the mRNA levels of the specific genes involved.[62] In that study, we detected recurrent amplicons for chromosomes 7 (50%), 12 (22%), 1 (11%), 4 (9%), 11 (4%), and 17 (4%), whereas homozygous deletions involved chromosomes 9p21 (52%) and 10q (22%). Most genes that displayed a high correlation between DNA copy number alteration (CNA) and mRNA levels were coded in the amplified chromosomes. For some amplicons, the impact of DNA CNA on mRNA expression was restricted to a single gene (eg, *EGFR* at 7p11.2), whereas for others, it involved multiple genes (eg, 11 and 5 genes at 12q14.1-q15 and 4q12, respectively). Although homozygous del(9p21) and del(10q23.31) included multiple genes, association between these DNA CNAs and RNA expression was restricted to a few genes [eg, the *MTAP* gene in case of del(9p21)].

Until now, the most comprehensive and reliable analysis of genomic alterations in GBM has been conducted by the TCGA Consortium. Initially, the TCGA Consortium published mRNA expression data and DNA CNA data on approximately 206 GBMs; at the same time, >600 genes were also sequenced in a subgroup of 91 GBMs.[24] Overall, three major signaling pathways were found to be consistently affected in many GBM tumors: the TKR-signaling pathway (88%), the TP53 pathway (87%), and the RB pathway (78%); the genes involved in these pathways included the *NF1* tumor-suppressor gene and the *PIK3R1* gene, which had been previously reported to be altered in GBM.[80] In a more recent multidimensional study including previous TCGA data, together with the patterns of somatic mutations, DNA CNA, and the tumor-associated GEP, an integrated molecular classification of GBM was proposed.[21] This classification included four subtypes of GBM defined by the patterns of alteration of the *EGFR*, *NF1*, *PDGFRA*, and *IDH1* genes and the GEP: classic, MQ, PN, and neural (Table 2). These subtypes also differed in their response to treatment and overall survival. Classic GBM displayed a characteristic profile with high proliferation, frequently associated with high-level *EGFR* amplification, monosomy 10, and homozygous del(9p21.3) targeting the *CDKN2A-p16*^{INK4a} gene. The MQ GBMs were defined by overexpression of MQ (CHI3L1/YKL40 and MET) and astrocytic (CD44 and MERTK) markers, plus *NF1* deletion or mutation (17q11.2). The PN GBMs presented with activation of both oligodendrocytic (*PDGFRA*, *OLIG2*, *TCF3*, and *NKX2-2*) and PN (*SOX*, *DCX*, *DLL3*, *ASCL1*, and *TCF4*) development-associated genes, and they were characterized by molecular

alterations of *TP53* and *PDGFRA*, as well as *PIK3CA/PIK3R1* and *IDH1* mutations. Finally, neural GBM lacked a distinctive genetic profile and display gene expression signatures that are similar to those found in normal brain tissue, with expression of neuron markers such as NEFL, GABRA1, SYT1, and SLC12A5. Although in primary GBM, several molecular abnormalities (eg, *EGFR*, *MTAP*, and *PDGFRA* alterations) were strongly associated with specific GEPs (eg, *EGFR* and *MTAP* genetic alterations were associated with PF, classic, and PN GBM, respectively),[21,99,100] the association between the genes involved in secondary GBM and their potentially associated GEP still remains to be determined and requires further assessment.

Conclusions

Overall, GBMs carry numerous recurrent genetic alterations that translate into different cytogenetic and genomic profiles that are difficult to distinguish on histopathological grounds. Although further validation in larger series of GBM is still needed, current knowledge about the complex genome of GBM points to the existence of four major profiles: classic, MQ, PN, and neural GBM. Such genomic profiles of GBM reflect both different cytogenetic profiles, as well as unique GEPs associated with distinct patterns of alteration of intracellular signaling pathways, in addition to key stages in neurogenesis and features of distinct normal central nervous system cell types. Additional pieces of evidence also confirm that the relationship between DNA (eg, copy number) alteration and gene expression at the mRNA level is not always straightforward; distant genetic interactions and epigenetic changes also have a significant impact on the expression of specific genes. Selection of just the most commonly amplified and mutated genes as a therapeutic target may not be sufficient. In turn, identification of driver mutations using large-scale, next-generation sequencing or single-tumor cell sequencing, as well as genomic analyses within individual tumors, in combination with new proteomic strategies to identify aberrant cancer peptides may contribute to the identification of new drugs and antibody targets that may help in suppressing the disease and improving the management of GBM patients.

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