

# Molecular biomarkers of glioblastoma: current targets and clinical implications

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**Abstract:** Recent genome-wide analysis of glioblastoma has revealed various molecular alterations that can be candidate targets of biomarker findings. Although glioblastomas are diagnosed on the basis of their histopathological morphological features, it has been demonstrated that molecular heterogeneity among glioblastomas is prominent, and pathological diagnosis cannot always predict the clinical behavior of the tumor. Thus, molecular biomarkers have been anticipated to provide prognostic and predictive significance. Given that recent medical treatment strategies have been progressing toward individualized therapy and many targeted drugs have been investigated, the identification of molecular biomarkers in glioblastoma will be of considerable therapeutic importance. Although the clinical implications of these biomarkers must be determined in prospective studies, a growing number of candidate biomarkers have been investigated. In this review, the recent molecular alterations in glioblastoma that may be significant biomarkers are summarized; particular focus is on loss of heterozygosity on chromosome 10, epidermal growth factor receptor (EGFR)/EGFR variant III expression, alterations in the receptor tyrosine kinase-phosphatidylinositol 3-kinase pathway, isocitrate dehydrogenase mutation, epigenetic alterations, gene expression profiling, and microRNA expression.

**Keywords:** *EGFRvIII*, *IDH*, global gene expression profiling

## Introduction

Glioblastoma (GBM) is the most malignant neoplasm in adults.<sup>1-3</sup> In recent years, many advancements have been made regarding treatment modalities for GBM patients. Fluorescence image-guided tumor removal with intraoperative neuro-functional monitoring increases the possibility of maximum tumor removal without neurological deficit, which prolongs patient survival.<sup>4,5</sup> Recent radiotherapy modalities such as intensity-modulated radiation therapy and heavy charged particle therapy have been investigated to improve the treatment efficacy of conventional radiation therapy.<sup>6,7</sup> Moreover, temozolomide (TMZ), a new oral alkylating chemotherapeutic agent, has been demonstrated to enhance patient survival,<sup>8,9</sup> and molecular targeted drugs such as epidermal growth factor receptor (EGFR) inhibitors and vascular endothelial growth factor receptor (VEGFR) antibodies have been used clinically.<sup>10</sup> However, despite this technical advancement of therapeutic modalities, the treatment of patients with GBM has only improved minimally, with a median survival time of approximately 14 months,<sup>11,12</sup> suggesting that effective therapeutic targets remain to be identified.

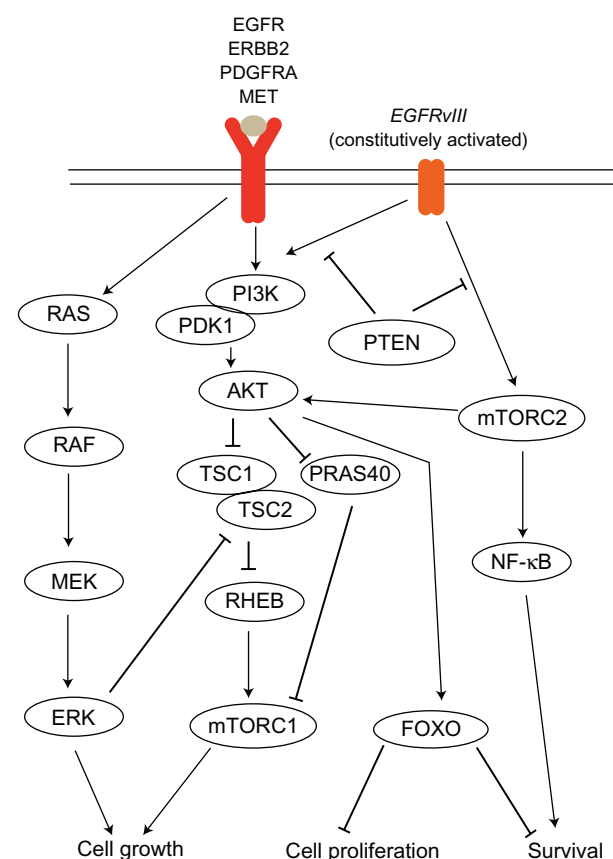
Recent molecular analysis has revealed characteristic molecular alterations in GBM that provide insight into its pathogenesis and tumor biology.<sup>13-19</sup> Moreover, technological

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advancements of genome-wide genomic and transcriptional analysis have shifted the biomarker development from local analysis to global molecular alterations. Recently, The Cancer Genome Atlas (TCGA) project performed integrated genome-wide analysis of DNA copy number, DNA methylation aberrations, gene expression, and nucleotide sequence aberrations in 206 GBMs.<sup>20</sup> This comprehensive analysis identified three critical signaling pathways in GBM – receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K) signaling, p53 signaling, and RB signaling – indicating that the genomic alterations of these pathways are associated with the acquired characteristics of tumor cells. As Hanahan and Weinberg describe in their excellent review, cancer cells acquire biological capabilities not observed in their normal counterparts.<sup>21</sup> The hallmarks of cancer cells include sustained proliferative signaling, resistance to growth suppression and cell death, replicative immortality, angiogenesis, and invasion and metastasis. In the most recent version, they added two emerging hallmarks to this original list, reprogramming energy metabolism and evading immune destruction, demonstrating that these hallmarks of cancer differentiate tumor cells from normal cells. Great efforts have been made in the cancer research field to identify meaningful molecular biomarkers from these molecular characteristics of cancer cells.

According to Biomarkers Definitions Working Group, a biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention,”<sup>22</sup> indicating that cancer biomarkers are functionally associated with the hallmarks of cancer. Biomarkers can be classified into diagnostic, prognostic, predictive, and pharmacodynamic subtypes.<sup>23</sup> Diagnostic biomarkers are characteristic biochemical and functional characters specific to cancers, and prognostic biomarkers can identify the patient groups with better clinical outcomes. Predictive biomarkers can be utilized to identify the patient groups that will benefit from a particular treatment. Pharmacodynamic biomarkers can measure a treatment effect in tumor cells. Growing numbers of studies have identified candidate biomarkers in various cancers including GBM.<sup>17,24–30</sup> For instance, chromosome 1p/19q codeletion is a prognostic and possible predictive biomarker of oligodendroglial tumors,<sup>24–26</sup> and O6-methylguanine-DNA methyltransferase (MGMT) methylation is a prognostic and predictive biomarker following TMZ treatment in GBM.<sup>31</sup> Because of the introduction of molecular targeted

drugs and individualized therapy for GBM treatment,<sup>32–35</sup> identification of meaningful molecular biomarkers is crucial for determining therapeutic strategy and predicting tumor recurrence. Moreover, a combination of biomarker genes has been reported to be more useful than a single biomarker.<sup>36</sup> Although the significance of biomarkers must be validated in prospective studies,<sup>37</sup> many candidate biomarkers of GBM have been published. In this review, some genetic, epigenetic, and transcriptional alterations that have been presumably designated as biomarkers in GBM are described as well as the emerging roles of cancer stem cell marker and microRNA (miRNA) expression.



**Figure 1** RTK-PI3K signaling pathway.

**Notes:** PI3K and RAS signaling is activated following ligand binding to EGFR and other RTKs such as ERBB2, PDGFRA, and MET. *EGFRvIII* signaling, which is constitutively activated without ligand binding, activates the mTORC2-NF-κB pathway independent of the PI3K-AKT pathway. Because PTEN suppresses PI3K and mTORC2 signaling, PTEN inactivation leads to the activation of these signaling pathways. AKT is activated following its phosphorylation by PDK1 and mTORC2. AKT phosphorylation leads to the activation of mTORC1 via inhibition of negative regulators of TSC1, TSC2, RHEB, and PRAS40. In addition, AKT inhibits forkhead (FOXO) transcription factors, resulting in cell proliferation and survival.

**Abbreviations:** EGFR, epidermal growth factor receptor; *EGFRvIII*, EGFR variant III; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; NF, nuclear factor; PI3K, phosphoinositide 3-kinase; PRAS40, proline-rich AKT substrate 40 kDa; PTEN, phosphatase and tensin homolog; RHEB, RAS homologue enriched in brain; RTK, receptor tyrosine kinase; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2.

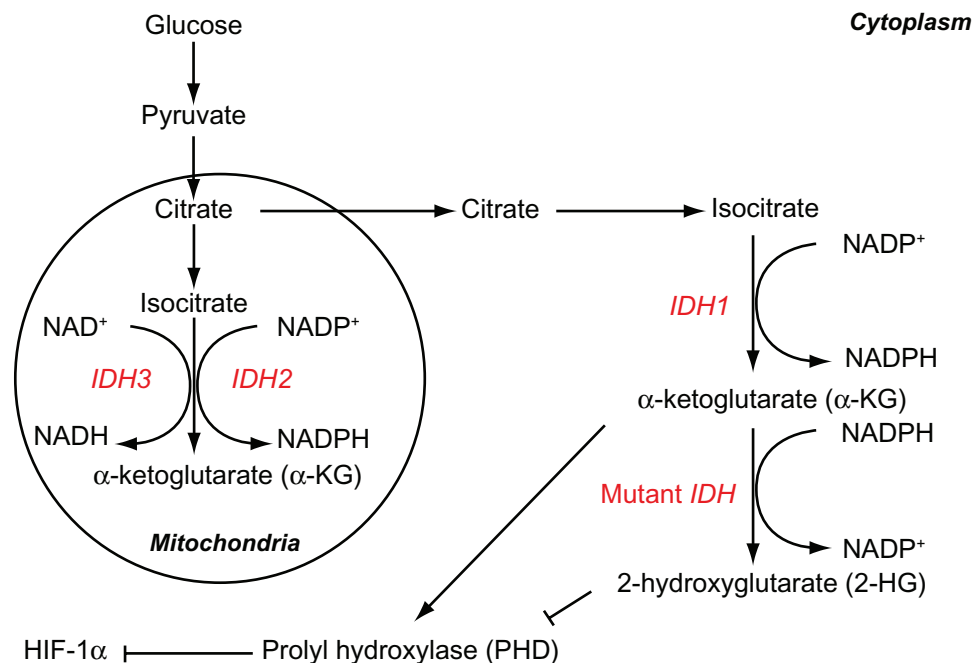
## Chromosome 10 loss of heterozygosity (LOH)

LOH is detected in many chromosomes in most common cancers.<sup>38,39</sup> In GBM, LOH is frequently observed at chromosomes 9p, 10, 17p, 19q, and 22,<sup>16</sup> but LOH at chromosome 10 is a critical genetic event. LOH can be routinely detected by polymerase chain reaction (PCR)-based polymorphic markers or fluorescence in-situ hybridization. Recently, genome-wide array-based technologies such as single nucleotide polymorphism-array analysis and comparative genomic analysis have been applied to detect copy number alterations.<sup>40,41</sup> Three commonly deleted loci have been identified thus far: 10p14-15, 10q23-24, and 10q25-pter.<sup>42,43</sup> Although LOH at 10p is also observed in low-grade glioma<sup>44</sup> and it is a rare event in secondary GBM, LOH at 10p and 10q regions has been reported in 60%–80% of cases of GBM, and these events can be genetic markers for both primary and secondary GBM.<sup>42,43,45,46</sup> In particular, LOH at 10q 25-pter is associated with acquisition of the GBM phenotype in secondary GBM.<sup>42</sup> By contrast, LOH at chromosome 10 is more extensive in primary GBM,<sup>43</sup> and the loss of an entire chromosome is common, suggesting that LOH of the entire chromosome 10 can be a molecular marker of primary GBM.

As the occurrence of specific frequent deletions is considered to indicate the presence of tumor suppressor genes implicated in tumor initiation and progression based on Knudson's two-hit theory, great efforts have been made to discover tumor suppressor genes on LOH loci. However, only phosphatase and tensin homolog (PTEN) has been identified in the 10q23 region as a critical tumor suppressor.<sup>47,48</sup> However, the mutation frequency is 15%–40%,<sup>49,50</sup> indicating the presence of other unidentified tumor suppressors. Nevertheless, LOH at chromosome 10q is a prognostic biomarker because GBM patients with 10q loss have a significantly shorter survival than patients who retain chromosome 10.<sup>45,51</sup> Although LOH of chromosome regions is normally attributable to deletion of one allele with copy number loss, it has been demonstrated that copy number-neutral LOH occurs frequently in GBM, indicating that this type of LOH can result from mitotic recombination or nondisjunction.<sup>52</sup>

## EGFR/EGFR variant III (EGFRvIII)

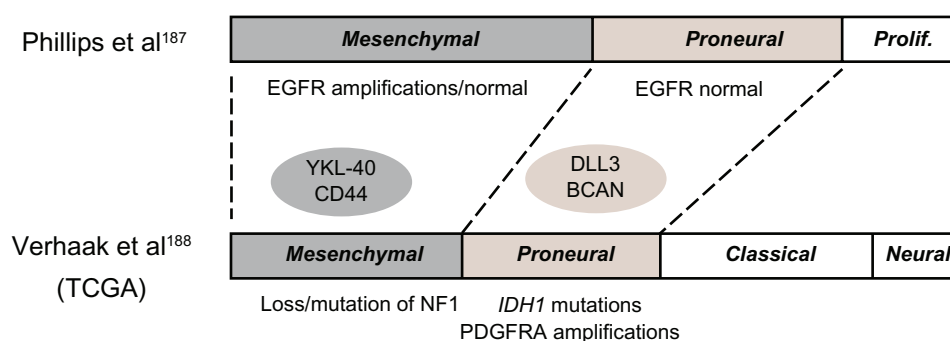
In GBM, the EGFR gene, which is located at chromosome 7, is amplified in 40%–50% of cases of GBM.<sup>20,53–55</sup> A recent statistical analysis of genomic alterations by Beroukhi et al demonstrated that there are two types of genomic



**Figure 2** The role of IDH and IDH mutations in cellular metabolism.

**Notes:** IDH1 is located in the cytoplasm, whereas IDH2 and IDH3 are located in mitochondria. IDH3 converts NAD<sup>+</sup> into NADH. Wild-type IDH1 converts NADP<sup>+</sup> into NADPH, which generates α-KG from isocitrate. Mutant IDH1 protein produces 2-HG from α-KG, thus mediating the formation of heterodimers between 2-HG and α-KG, which inhibit the activities of α-KG-dependent PHD. The suppression of PHD leads to the increased expression of HIF-1.

**Abbreviations:** HG, hydroxyglutarate; HIF, hypoxia inducible factor; IDH, isocitrate dehydrogenase; KG, ketoglutarate; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; PHD, prolyl hydroxylase.



**Figure 3** Transcriptional subtypes of glioblastoma.

**Notes:** Mesenchymal and proneural subtypes are concordant between the study by Phillips et al<sup>187</sup> and that by Verhaak et al.<sup>188</sup> In the Phillips et al study, EGFR amplification was frequently associated with the mesenchymal subtype, whereas the EGFR locus was likely to be normal in the proneural group. The Verhaak et al study showed that the mesenchymal subtype is associated with loss/mutation of NF1 and *IDH1* mutation, and proneural subtype is associated with PDGFRA amplification.

**Abbreviations:** EGFR, epidermal growth factor receptor; *IDH1*, isocitrate dehydrogenase; NF, nuclear factor; TCGA, The Cancer Genome Atlas.

amplification of chromosome 7 – focal high-level amplification at the EGFR gene, which is associated with EGFR activation, and broad low-level amplification of the entire chromosome, which often activates another receptor tyrosine kinase, MET – suggesting that the functional significance of EGFR amplification needs to be determined in the context of the level of amplification.<sup>40</sup> EGFR amplification is often associated with truncated mutations, and *EGFRvIII* is the most common mutant.<sup>56</sup> *EGFRvIII* is detected in 50%–60% of cases of GBM with amplified wild-type EGFR, and this mutant has deletions of exon 2–7, lacking a portion of the extracellular ligand binding domain, thus constitutively activating downstream pathways without ligand binding.<sup>57,58</sup> Activating signaling pathway via wild-type EGFR overexpression can be differentiated from EGFR-non-overexpressing tumors, and EGFR-amplified tumors have a worse prognosis than nonamplified tumors.<sup>59</sup> However, *EGFRvIII* expression has been demonstrated to confer a worse prognosis than EGFR wild-type expression alone.<sup>60,61</sup> Transfection of the *EGFRvIII* mutant into GBM cell lines enhances tumorigenicity in vivo due to increased proliferation and reduced apoptosis.<sup>62,63</sup> Furthermore, it has been demonstrated that the expression of the constitutive mutant form of *EGFRvIII* in glial or neural cells can induce GBM formation in mice via interactions with another signaling pathway, and the genetic characterization of the formed tumors recapitulated human GBM.<sup>64,65</sup> Although the downstream pathways activated by *EGFRvIII* have not been completely elucidated, *EGFRvIII* is constitutively phosphorylated, thus resulting in stronger PI-3K (discussed in the next section) activation than wild-type EGFR signaling.<sup>66,67</sup> Moreover, it has been reported that *EGFRvIII* confers enhanced tumorigenicity and therapeutic resistance through the Ras-Shc-Grb2, interleukin-8, and mammalian

target of rapamycin (mTOR) complex 2 (mTORC2)-nuclear factor (NF)- $\kappa$ B pathways.<sup>68–70</sup> These results indicate that *EGFRvIII* uses different downstream signals from wild-type EGFR, suggesting that this variant can be a molecular target for individualized therapy. Indeed, it has been reported that mutant *EGFRvIII* expression can be a molecular predictor of a better response to EGFR kinase inhibitor therapy in GBM.<sup>71</sup> In addition, Montano et al recently demonstrated that *EGFRvIII* is a molecular predictor of longer overall survival in GBM patients treated with surgery followed by adjuvant radiotherapy and TMZ in contrast to earlier studies in which *EGFRvIII* was a biomarker of a poorer prognosis.<sup>72</sup> This discrepancy may be partially explained by the fact that Montano et al used a reverse-transcription PCR assay on selected regions of formalin-fixed, paraffin-embedded tumor specimens, which is a more sensitive method for detecting *EGFRvIII* expression than conventional immunohistochemistry. More importantly, the patient population in the Montano study was uniform in that all patients underwent surgery followed by concomitant radiation and TMZ therapy, which is the consensus standard therapy that was introduced recently in the clinic, indicating that the biological significance of *EGFRvIII* expression should be explored in the context of therapeutic modalities. Furthermore, Bredel et al recently identified a subset of GBM that harbors monoallelic loss of NF $\kappa$ BIA, which encodes I $\kappa$ B, a critical negative regulator of the canonical NF- $\kappa$ B pathway, and NF $\kappa$ BIA deletion and EGFR amplification are mutually exclusive. This study also implied a functional relationship between EGFR and NF- $\kappa$ B signaling and potential therapeutic targets.<sup>73</sup>

Given that *EGFRvIII* expression is not found in any normal tissues and it is a constitutively active cell surface protein, *EGFRvIII* can also be a target for peptide vaccination immu-

notherapy.<sup>74,75</sup> The recent data of a Phase II multicenter clinical trial revealed that *EGFRvIII*-targeted vaccination therapy is a promising therapy for patients with GBM.<sup>76</sup> Regarding EGFR-targeted therapy, GBM patients respond poorly to EGFR kinase inhibitors such as gefitinib and erlotinib in contrast to lung cancer patients with EGFR kinase domain mutations.<sup>77</sup> Recently, novel oncogenic missense mutations in the extracellular domain and carboxyl-terminal deletion mutants have been reported, and these specific mutants selectively respond to EGFR kinase inhibitors.<sup>78–80</sup> These results imply that similar to the observations in lung cancer, EGFR-specific mutations are associated with a good response to EGFR kinase inhibitors in GBM. Further studies are mandatory to address this issue.

## RTK/PI3K pathway

As has been reported in various studies, the PI3K-AKT pathway is frequently activated in GBM, indicating that it can be a molecular target.<sup>81–83</sup> Indeed, many newly developed drugs targeting the PI3K pathway have been investigated in clinical trials for various types of cancers, including GBM.<sup>84</sup> This pathway is known to be activated through several mechanisms, including RTK amplifications, and mutations including EGFR as described previously (Figure 1). The PI3K complex consists of a catalytically active protein, p110a, encoded by PIK3CA, and a regulatory protein, p85a, encoded by PIK3R1. Frequent active mutations of PIK3CA in GBM have been reported,<sup>85</sup> and TCGA analysis revealed somatic mutations in PIK3R1,<sup>20</sup> indicating that PIK3CA and PIK3R1 mutations are mechanisms of PI3K pathway activations. However, AKT1 mutations have been reported to be absent in GBM.<sup>86</sup> PTEN, which maps to chromosome 10q23, has been identified as a tumor suppressor gene in GBM.<sup>47,48</sup> PTEN is a negative regulator of the PI3K pathway, and inactivation of PTEN via genetic loss, mutation, and epigenetic mechanism occurs in 30%–40% of cases of GBM. This inactivation leads to PI3K pathway activation.<sup>87</sup> It has been reported that PTEN inactivation confers resistance to EGFR kinase inhibitors in patients harboring *EGFRvIII* mutations by activating the downstream AKT pathway and increasing EGFR activity by impairing degradation.<sup>71,88</sup> In addition to its function as a regulator of cell proliferation and migration through lipid phosphatase function, PTEN is reported to be a key player in the DNA damage response and stem cell maintenance. Although PTEN protein has been assumed to be localized in the cytoplasm, growing amounts of evidence indicate that PTEN is primarily localized in the nucleus in differentiated and resting cells, and its predominant

expression is shifted to the cytoplasm in cancer cells, thereby suggesting that PTEN can translocate from the nucleus to the cytoplasm.<sup>89</sup> It has been also revealed that nuclear PTEN plays an essential role in maintaining chromosome integrity, partly through regulating double-strand break repair pathways.<sup>90</sup> Moreover, Li et al revealed that PTEN loss and *EGFRvIII* synergistically transform neural precursor cells by inducing chromosome instability,<sup>91</sup> and McEllin et al have demonstrated that PTEN loss compromises the homologous recombination repair pathway in astrocytes.<sup>92</sup> These results demonstrate that lipid phosphatase-independent roles of PTEN also have an important role in gliomagenesis.

mTOR is another molecular target of the PI3K pathway. mTOR exists in 2 distinctive complexes with other molecules.<sup>93</sup> mTOR complex 1 (mTORC1) forms a complex with regulatory associated protein of mTOR (Raptor) and mLST8 and is an important downstream effector of the PI3K pathway, acting as a signal integrator of nutrients, growth factors, and energy and stress inputs to involve the processes of protein and lipid biosynthesis and cellular metabolism.<sup>94</sup> This indicates that mTORC1 can be a therapeutic target of rapamycin and its derivatives. However, it has been demonstrated that single mTOR inhibitor therapy does not have significant efficacy in GBM patients due to the activation of a negative-feedback AKT pathway, suggesting a rationale for combination therapy with mTOR inhibitors and PI3K inhibitors.<sup>95</sup> In contrast to mTOR1, the roles of rapamycin-insensitive mTORC2 have been elusive. Recently, Tanaka et al demonstrated that *EGFRvIII* signaling activates the mTORC2-NF- $\kappa$ B pathway, indicating a critical role of mTORC2 in chemoresistance mechanisms independent of the AKT pathway.<sup>70</sup> This study clearly demonstrated that both mTORC1 and mTORC2 play important roles in gliomagenesis and therapeutic resistance. Further studies have been undertaken to gain more insight into mTOR signaling in GBM.

## p53 and Rb pathways

The tumor suppressor TP53 is a key molecule that plays critical roles in various biological processes such as the cell cycle, DNA damage response, apoptosis, and cell differentiation.<sup>96,97</sup> The function of p53 is negatively regulated by MDM2 and MDM4, the expressions of which are subject to negative regulation by ARF. This p53/MDM2/4/ARF signaling is altered in 87% of TCGA cases.<sup>20</sup> TP53 mutation is detected in 31%–35% of all GBM, although TP53 mutation is more frequently observed in secondary GBM (65%) than in primary GBM (28%).<sup>20,98</sup> Amplification of MDM2



is detected in approximately 10% of GBM exclusively, without TP53 mutation.<sup>20,98,99</sup> Loss of ARF expression, which can be induced by homologous deletion, mutation, and promoter methylation, has been observed at a frequency of 49%–76% in GBM.<sup>15,20</sup> Regarding the prognostic significance of the TP53 signaling pathway, TP53 status has been reported to be neither a prognostic nor a predictive biomarker in GBM.<sup>24,100</sup> However, owing to the crucial role of the p53 signaling pathway in numerous cancers including GBM,<sup>13,14</sup> efforts have been made to develop therapeutic strategies that target the p53 pathway by reactivating p53 signaling with peptides and small molecules.<sup>101,102</sup>

The retinoblastoma (*RB*) gene is a negative regulator of cell cycle progression from G1 to S phase.<sup>13,14</sup> Inactivation of the *RB* gene by homologous deletion and mutation is observed at a frequency of 11%.<sup>20</sup> The rate of methylation of the promoter region in the *RBI* gene has been reported to be 25%.<sup>103</sup> The *RB* gene signaling pathway is altered in 78% of GBM cases<sup>20</sup> owing to the amplification of cyclin-dependent kinase 4 (CDK4) and loss of p16INK4A, as well as altered expression of the *RBI* gene. Although the importance of the inactivation of the *RB* pathway in glioma progression has been demonstrated, the prognostic significance of alterations in the *RB* pathway is controversial.<sup>100,104</sup>

## Isocitrate dehydrogenase (*IDH*) mutation

Genome-wide mutational analysis disclosed recurrent mutations in the active sites of the *IDH* 1 or 2 gene in GBM at a frequency of 12% (18/149).<sup>105</sup> The most common sites of mutations in *IDH1* and *IDH2* are codons R132 and R172, respectively. Secondary GBM has a remarkably high frequency of *IDH* mutations compared with primary GBM (5/6 versus 7/99), and patients with *IDH* mutations have a significantly improved prognosis. Subsequent analysis of a large set of glioma samples with different histologies and grades has also demonstrated that *IDH* mutations occurred in 70%–100% of World Health Organization-grade II–III astrocytic tumors, oligodendroglial tumors, and secondary GBM, whereas primary adult GBM exhibited a low frequency of this mutation (4.9%).<sup>106–108</sup> These results implicate a role of *IDH* mutations in gliomagenesis, and these mutations are associated with the malignant progression of gliomas. The low frequency of mutation in primary GBM indicates the presence of *IDH*-dependent and *IDH*-independent pathways of glioma development.

*IDH1* is localized in the cytoplasm and peroxisomes, whereas *IDH2* and *IDH3* are localized in the mitochondria,

where they participate in the citric acid cycle for energy production.<sup>109–111</sup> Wild-type *IDH1* and *IDH2* function as NADP<sup>+</sup>-dependent *IDHs* that catalyze the oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG), generating NADPH from NADP<sup>+</sup> (Figure 2). In normal cells, mitochondrial NADPH is of fundamental importance in the defense against reactive oxygen species (ROS), regulating NADPH-dependent glutathione reductase. Different from the wild-type proteins, mutant *IDH1* and *IDH2* proteins display the novel enzymatic property of producing 2-hydroxyglutarate (2-HG) from  $\alpha$ -KG, facilitating heterodimer formation between 2-HG and  $\alpha$ -KG.<sup>112–114</sup> The dominant-negative effect of 2-HG and the reduction of  $\alpha$ -KG inhibit the activities of  $\alpha$ -KG-dependent dioxygenase family proteins (prolyl hydroxylase) that catalyze the hydroxylation of hypoxia inducible factor (HIF) to cause its degradation. Thus,  $\alpha$ -KG downregulation increases the levels of HIF-1 $\alpha$ , a transcription factor that facilitates tumor growth when oxygen is low.<sup>115</sup> *IDH* is the first metabolic enzyme for which genetic and biochemical alterations have been incorporated into the basics of cancer biology. Given that tumors with *IDH1* and *IDH2* mutations have highly elevated (10–100-fold) levels of 2-HG, 2-HG can be used as an important biomarker of tumors with *IDH1/2* mutations.<sup>112,113</sup> Choi et al recently reported that 2-HG can be detected using magnet resonance spectroscopy (MRS) in *IDH*-mutated gliomas, suggesting that the noninvasive detection of 2-HG may prove to be a valuable diagnostic biomarker.<sup>116</sup>

An emerging role of *IDH1* and *IDH2* mutations is their association with the hypermethylation phenotype (discussed in the next section) in tumor cells. In leukemia cells, mutant *IDH1* and *IDH2* disrupt the function of TET2, a DNA demethylase enzyme, causing DNA hypermethylation and impairing differentiation in hematopoietic cells.<sup>117</sup> Although genomic mutation of TET2 is not evident in glioma, *IDH* mutations are significantly associated with a methylator phenotype (glioma-CpG island methylator phenotype [G-CIMP] phenotype) in GBM.<sup>118</sup> The functional correlation between *IDH* mutations and the G-CIMP phenotype was verified by the recent study by Turcan et al, who demonstrated that *IDH* mutation is the cause of the G-CIMP phenotype through an epigenomic reprogramming mechanism.<sup>119</sup> A subsequent study demonstrated that *IDH* mutations are associated with dysregulation of glial differentiation and global histone methylation in low-grade gliomas, and 2-HG can inhibit histone demethylation, blocking the differentiation of nontransformed cells.<sup>120</sup> These results indicate that *IDH* mutants have a wide variety of biological functions.

Along with their functional significance, *IDH* mutations are prognostic biomarkers in glioma patients. It has been revealed that patients with *IDH1/2* mutations have a significantly better outcome than those with wild-type *IDH* in GBMs, anaplastic astrocytomas, and anaplastic oligodendroglial tumors.<sup>105–107,121,122</sup> Although the difference is not significant, patients with diffuse astrocytoma with *IDH1/2* mutations tend to have a longer survival than those with wild-type *IDH*.<sup>123</sup> As specific mutant antibodies against R132 *IDH1* and R176 *IDH2* are available in the clinical setting, immunohistochemical detection of *IDH* mutations, as well as neuroradiological detection by MRS as described above, can facilitate patient stratification for selecting patients with a better prognosis. Great efforts have been made toward developing targeted therapies for mutant *IDH1* and *IDH2* that modify their enzymatic activity. However, these targeted therapies have potential effect only for a small subset of primary GBM because pathway activation in most primary GBM subtypes is *IDH* independent.

## Epigenetic alterations

“An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence;” this results in variable gene expression. Epigenetic modifications in cancer are characterized by global changes in DNA methylation and altered histone modifications.<sup>124–126</sup> Emerging roles of epigenetic alterations in glioma biology have been recently reported.<sup>27,28,118,127–129</sup> DNA methylation occurs at the 5′ position of the cytosine ring within CpG dinucleotides, resulting in gene silencing. DNMT1 is the DNA methyltransferase that maintains existing methylation during DNA replication, whereas DNMT3A and DNMT3B are responsible for methylating previously unmethylated regions. In cancer cells, epigenetic alterations of DNA methylation are present with global DNA hypomethylation and hypermethylation of locus-specific promoters within CpG islands. In GBM, it was reported that global hypomethylation associated with genome-wide reduction of 5-methylcytosine was detected in 8 of 10 primary GBM tissues, and the extent of 5-methylcytosine reduction was at least 60%–70% with more severe reduction to less than 50% in two cases.<sup>130</sup> DNA hypomethylation mainly occurs at repetitive DNA sequences, generating chromosome rearrangement, reactivation of transposable elements, and loss of imprinting.<sup>131</sup> Indeed, Fanelli et al demonstrated that demethylation of repetitive sequences such as tandem repeat satellite 2 located at the juxacentromeric region of chromosome 1 could contribute to genomic instability in

human GBM.<sup>132</sup> Furthermore, loss of imprinting of insulin-like growth factor-2 is present in more than half of gliomas, suggesting a critical role of this protein in the development of glioma.<sup>133</sup>

Locus-specific hypermethylation of CpG islands in the promoter region is associated with the maintenance of tumor suppressor gene silencing as well as other various functions such as cell cycle regulation, DNA repair, angiogenesis, and invasion. CpG islands in the promoters of tumor suppressor p53, PTEN, RB, CDKN2/p16, and P14ARF are frequently methylated in GBM, indicating that their tumor suppressive function is inhibited.<sup>127,129</sup> In addition, the DNA repair protein MGMT is frequently methylated in the promoter regions. MGMT removes TMZ-generated O6-methylguanine DNA adducts by transferring the methyl adducts to its own cysteine residues, indicating that MGMT activity is associated with the therapeutic response to TMZ.<sup>134–136</sup> As promoter methylation is likely to be associated with MGMT gene silencing,<sup>137,138</sup> the potential utility of promoter methylation of MGMT as a predictive biomarker for TMZ chemotherapy has been investigated. Consequently, it was demonstrated that MGMT methylation is associated with a better clinical response to TMZ and concomitant radiotherapy in GBM than wild-type MGMT.<sup>11,31,139,140</sup> However, this notion was challenged by the finding that MGMT inactivation was associated with a better response to radiation therapy alone, suggesting that the functional significance of MGMT methylation needs to be revalidated in future studies.<sup>141</sup> Additionally, Kreth et al demonstrated that the MGMT mRNA expression level is not consistent with the degree of promoter methylation in 20% of cases of GBM, and mRNA expression is a better predictor of the efficacy of concomitant radio-chemotherapy than MGMT methylation.<sup>142</sup> Although it has not been completely determined whether promoter methylation, mRNA expression, or the protein level best predict the therapeutic response, MGMT gene silencing is associated with chemotherapeutic resistance, but it is not the only predictor.<sup>143</sup> Recently, it was reported that the base excision repair enzyme alkylpurine-DNA-*N*-glycosylase (APNG), which repairs the major cytotoxic lesions N3-methyladenine and N7-methylguanine, confers TMZ resistance independent of MGMT methylation, and APNG expression is also regulated by promoter methylation.<sup>144</sup> Collectively, these results indicate that CpG island hypermethylation in DNA repair genes can be a biomarker in GBM.

A CIMP was first characterized in subsets of colon cancer tumors.<sup>145</sup> TCGA group recently reported that a subset of GBM displays hypermethylation of a large number of loci, a

characteristic called a G-CIMP.<sup>118</sup> G-CIMP defines a distinct subgroup of gliomas that belong to the proneural type (described in the next section) and that are tightly associated with *IDH* mutations. Thus, it is possible that G-CIMP status can stratify the patients with different molecular features and clinical responses to therapeutic interventions.

Gene expression is regulated by histone modifications such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination.<sup>124–126</sup> Histones are small proteins with a high proportion of positively charged amino acids (lysine and arginine) consisting of two molecules each of H2A, H2B, H3, and H4. Because histone acetylation neutralizes the positive charge of lysine residues and weakens the binding between DNA sequences, histone acetylation is linked to transcriptional activation. Meanwhile, histone methylation mainly occurs on the side chains of lysine and arginine, and it activates (H3K4me2, H3K4me3) or represses (H3K9me2, H3K27me3) transcription depending on which site is methylated. Histone deacetylase (HDAC) catalyzes the deacetylation of lysine residues, resulting in transcriptional repression. Given that HDAC is overexpressed in cancer cells, HDAC inhibitors (HDACi) have been tested for clinical utility in GBM.<sup>127,128</sup> The basic mechanism of HDACi therapy in cancer treatment is that they relax the chromatin structure, thus allowing access of DNA damaging agents to chromatin, which results in the reversal of epigenetic tumor suppressive gene silencing. Although several classes of HDACi compounds are available, such as hydroxamates (SAHA, TSA) and aliphatic acids (valproic acid), no single agent is effective against GBM thus far. The clinical implication of histone modification and the utility of therapeutic targets should be evaluated in future research.

## Stem cell marker expression

Recent cancer stem cell biology findings advocate that rare populations of cancer stem cells define therapeutic response to chemo-radiotherapy, and they can be a source of tumor recurrence.<sup>146–149</sup> Glioma stem cells have been identified and propagated from tumor cell populations,<sup>150,151</sup> and the glioma stem cell paradigm has been reviewed in many articles.<sup>152–157</sup> It has been demonstrated that glioma stem cells exhibit therapeutic resistance to chemo-radiotherapy,<sup>158–160</sup> indicating that glioma stem cells can be therapeutic targets.<sup>161–163</sup> Because glioma stem cells are known to express neural stem cell markers,<sup>164–166</sup> these markers can be used to isolate tumor cells with stem cell properties. In addition, the expression of some stem cell markers such as CD133,

Nestin, BMI-1, MELK, and Notch in GBM tissue has been reported to have prognostic significance in GBM.<sup>167–173</sup> However, it should be remembered that the stem cell marker expression of tumor cells does not indicate its stem cell potential. Moreover, increasing amounts of evidence have revealed that the therapeutic resistance of glioma stem cells is defined by both the inherent features of glioma stem cells and close interaction with the tumor microenvironment, suggesting that the functional significance of glioma stem cells should be discussed in the context of the tumor microenvironment.<sup>174–178</sup>

## Global gene expression profiling

Recent technological advancements in genome-wide expression profiling have demonstrated that the molecular stratification of GBM provides better prognostic information than traditional histopathological classification, suggesting that this type of molecular subclassification can be a better classifier in terms of clinical implications.<sup>179–183</sup> Thus, many attempts have been made to identify biomarkers using global expression profiling.<sup>184</sup> Although the statistical variability of global expression data is of concern in some situations,<sup>185,186</sup> several clinically relevant subclassifications have been advocated. Phillips et al identified three GBM subtypes (proneural, proliferative, and mesenchymal) based on the expression profiles of genes correlated with patient survival.<sup>187</sup> The proneural subtype exhibits high expression of genes implicated in neurogenesis and those associated with better clinical outcomes. By contrast, the proliferative and mesenchymal subtypes are characterized by the high expression of genes correlated with cell proliferation and angiogenesis, respectively, and both subtypes are associated with poor clinical outcomes. Verhaak et al classified TCGA GBM samples into four subtypes (proneural, neural, mesenchymal, and classical) via consensus clustering of genome-wide expression profiles.<sup>188</sup> In this subclassification scheme, aberrations and the gene expression of *EGFR*, *NF1*, and *PDGFRA/IDH1* each define the classical, mesenchymal, and proneural subtype, respectively. However, no prognostic differences were observed among the four different subtypes. Although the classification schemes by Phillips et al<sup>187</sup> and Verhaak et al<sup>188</sup> used different sample sets and methodologies, Huse et al<sup>189</sup> used cross-validation analysis to reveal that the proneural and mesenchymal signatures are concordant between the two studies, suggesting that these two subcategories are robust (Figure 3). The proneural subtype displays frequent associations with the G-CIMP phenotype



and secondary GBM, whereas the mesenchymal subtype is exclusively detected in primary GBM. In addition, it has been demonstrated that tumors tend to shift toward mesenchymal signaling upon recurrence. Therefore, the mesenchymal signaling pathway can be a therapeutic target. YKL-40 has been designated as a representative mesenchymal marker.<sup>187,188</sup> Carro et al identified two transcriptional factors, C/EBP $\beta$  and STAT3, as initiators and master regulators of mesenchymal transformation.<sup>190</sup> Furthermore, Bhat et al revealed that transcriptional coactivator with PDZ-binding motif (TAZ) regulates mesenchymal differentiation in glioma.<sup>191</sup> Although many other signaling molecules implicated in this pathway remain to be identified, it is possible that inhibiting these key signaling molecules can have a tumor-suppressive effect. In addition, Prins et al reported that mesenchymal gene signatures may identify an immunogenic subgroup of GBM that may be more responsive to dendritic immunotherapy,<sup>192</sup> suggesting that the mesenchymal signature can be a predictive molecular marker for several therapeutic modalities.

## miRNA expression

miRNAs are 21-nucleotide small, noncoding RNA molecules that regulate the expression of a wide variety of genes at the post-transcriptional level.<sup>193–195</sup> Emerging evidence indicates that miRNAs can function as negative gene regulators in normal tissues and as tumor suppressors and oncogenes in various cancers.<sup>196–198</sup> MiR-21 is a highly expressed miRNA in cancers including GBM, and it acts as an oncogene to inhibit apoptosis in GBM cells.<sup>199,200</sup> Several other miRNAs have been validated as having functional significance in GBM biology, including miR-7, miR-34a, miR-124, miR-137, and miR-128.<sup>201–205</sup> Recently reported is that miR-196 might play an important role in the malignant progression of GBM and have prognostic significance.<sup>206</sup> Given that miRNAs have the potential to regulate the expression of a large number of genes, growing evidence indicates that miRNA expression profiles are better classifiers than messenger RNA-based classification in terms of clinical implications in cancer patients.<sup>207</sup> Srinivasan et al recently demonstrated that a 10-miRNA expression signature predicts patient survival in GBM patients.<sup>208</sup> Kim et al performed consensus clustering of GBM samples using miRNA expression profiles and identified five clinically and genetically distinct subclasses of GBM that are related to a different precursor cell type with robust survival difference.<sup>209</sup> These results indicate that miRNAs are useful for subclassifying GBMs and have potential utility as molecular biomarkers.

## Future perspectives

Although the current status of molecular biomarkers has been discussed by focusing on DNA, mRNA, and miRNA in this review article, a growing number of studies have been published that describe the application of proteomics to biomarker discovery in glioma patients.<sup>210</sup> Because biological fluids such as plasma/serum and cerebrospinal fluid are readily accessible sources for biomarker development, it can be confidently anticipated that many meaningful molecular biomarkers will be developed by less-invasive testing. Recently, the levels of serum YKL-40, a mesenchymal marker, were reported to be correlated with radiological disease status and patient survival in high-grade glioma, suggesting that serum YKL-40 is a prognostic and predictive molecular biomarker in GBM patients.<sup>211</sup> In addition, recent evidence has revealed that tumor miRNA is secreted as circulating miRNA into body fluids, and this miRNA can be designated as a new potential biomarker for cancer diagnosis and prognosis.<sup>212,213</sup>

Exosomes (microvesicles) are membranous vesicles of 30–100 nm diameter that are released from many cell types into the extracellular space. A recent study showed that GBM tumor cells secreted exosomes that could be detected in the serum of patients. Moreover, these secreted exosomes contained tumor-specific mRNAs, miRNAs, and signaling proteins, indicating that tumor exosomes could be a potential source of novel biomarkers.<sup>214,215</sup> Indeed, tumor-specific *EGFRvIII* was detected in serum exosomes in GBM patients, and the level of *EGFRvIII* correlated with tumor removal.<sup>214</sup> Thus, exosomes are expected to be a biological source for biomarker development using serum drawn from GBM patients.

## Concluding remarks

Numerous molecular features have been identified as candidate biomarkers of GBM. As cancer treatments including GBM have been moving toward individualized therapy, identifying predictive biomarkers is requisite for targeted therapy in GBM, and the clinical implications of these biomarkers should be critically evaluated. In addition, the clinical utility of a combination of multiple markers should be pursued. Furthermore, as stated previously, noninvasive biomarker findings in body fluids such as serum and cerebrospinal fluid have demonstrated promising results.<sup>211–215</sup> It can be envisaged that new validated biomarkers of GBM will be used clinically to stratify patients and to derive prognostic and predictive information.

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## Disclosure

The authors report no conflicts of interest in this work.

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