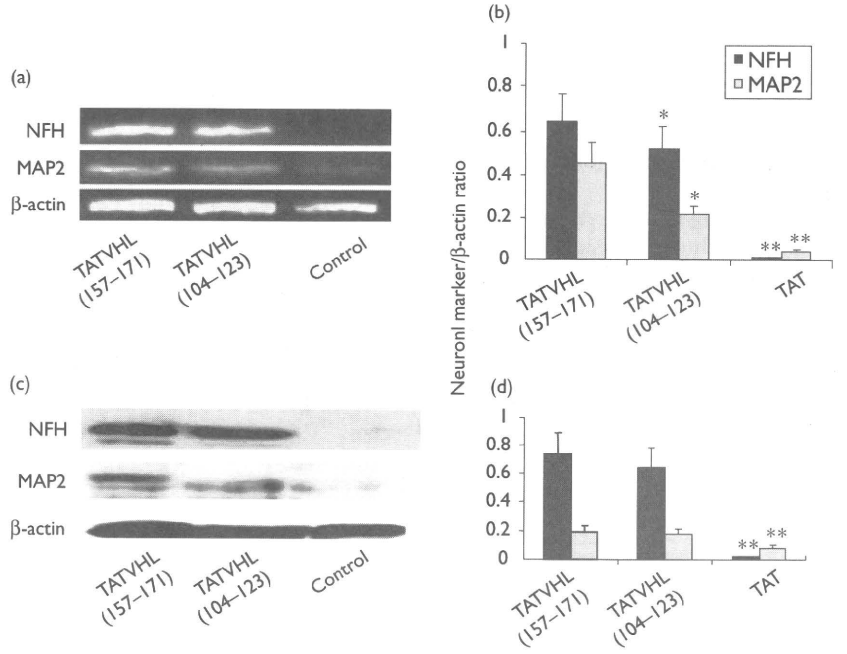
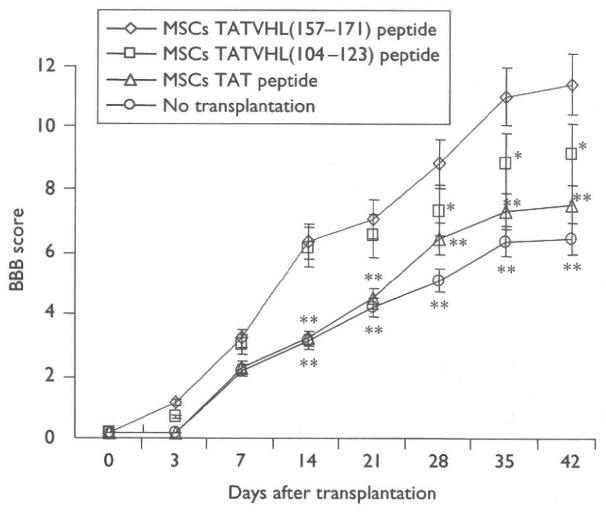
Fig. 2



Western blotting (a and b) and reverse transcriptase polymerase chain reaction analyses (c and d) for NFH and MAP2 in TATVHL(157–171) peptide-treated, TATVHL(104–123) peptide-treated, or TAT peptide-treated bone marrow stromal cells (MSCs) 7 days after the treatment. The results show TATVHL(157–171)-peptide-treated MSCs show significantly greater expression of NFH and MAP2 than TATVHL(104–123) or TAT peptide-treated MSCs at both mRNA and protein levels. *$P<0.05$; **$P<0.01$ in significant difference between TATVHL(157–171) and TATVHL(104–123) or TAT.

Fig. 3



Basso–Beattie–Bresnahan (BBB) scores for assessing recovery of behavioral performance after spinal cord injury in the various groups. The BBB score was significantly improved for the rats transplanted with TATVHL(157–171) peptide-treated bone marrow stromal cells (MSCs) compared with the score for the nontransplantation control. *$P<0.05$; **$P<0.01$ in significant difference between TATVHL(157–171) and TATVHL(104–123) or TAT.

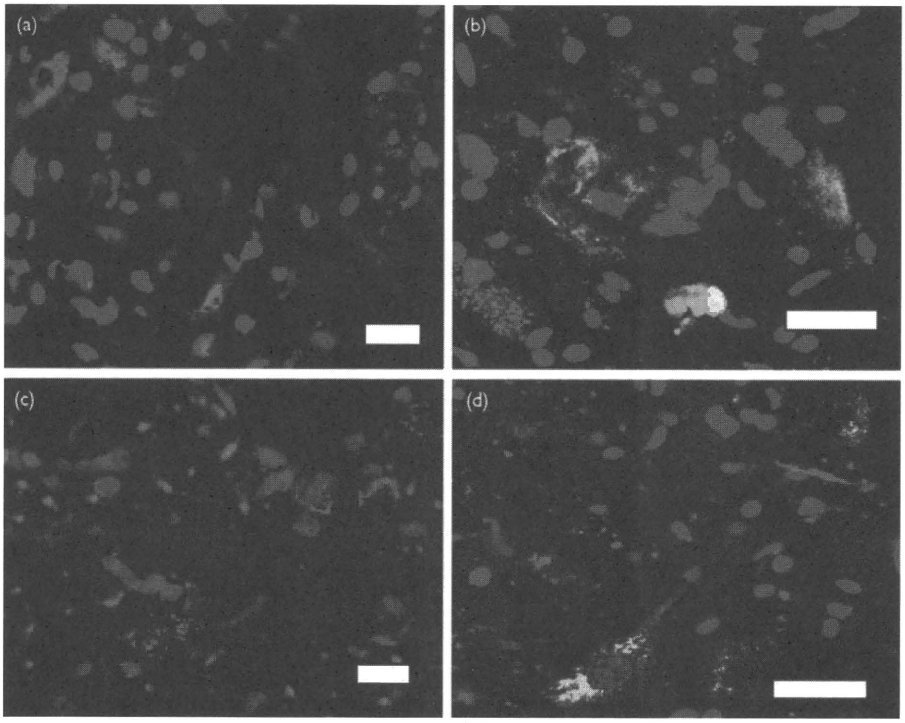
greater than that of TAT peptide-delivered cells ($2.3 \pm 0.6\%$) ($P < 0.01$). In addition, PKH-prelabeled MSCs had co-localized with NFH in $28.8 \pm 3.5\%$ of TATVHL(157–171) peptide-delivered cells whereas those did with NFH in $6.3 \pm 1.4\%$ of TAT peptide-delivered cells, indicating that TATVHL(157-171) peptide MSCs had differentiated more into NFH-positive cells than TAT peptide-delivered MSCs (Fig. 4).

Discussion

Neuronal differentiation of MSCs before cell transplantation is fundamental for therapy aimed at regeneration. In this study, we proposed a novel method using intracellular delivery of VHL peptide for neuronal differentiation in MSCs. Compared with neural progenitor/stem cells [8,9] or skin-derived precursors [10], MSCs showed less neuronal differentiation by intracellular delivery of VHL peptide. Although the mechanism of neuronal differentiation by intracellular delivery of VHL peptide is unclear, the response to VHL peptide might be different in cell types.

Our transplantation experiment has shown that untreated naive MSCs were unsatisfactorily differentiated into neuron-like cells and were insufficient to recover the behaviour of the spinal cord injury model rats, whereas VHL peptide-delivered MSCs were significantly more

Fig. 4



Confocal immunohistochemical images of nontreated or treated bone marrow stromal cells engrafted in injured rat spinal cord sites. The transplanted cells were prelabeled with red fluorescence PKH. The cells were stained with cell-specific markers and their nuclei were stained with DAPI. (a) Image of NeuN (green), PKH (red), and DAPI (blue) in the nontreated cells engrafted tissue. (b) Image of NeuN in the treated cells engrafted tissue. (c) Image for NFH (green), PKH (red), and DAPI (blue) in the nontreated cells engrafted tissue. (d) Image for NFH (green), PKH (red), and DAPI (blue) in the treated cells engrafted tissue. Scale bar = 10 μm.

differentiated into neuron-like cells and were sufficient to recover the behaviour. These results suggest that neuronal differentiation of MSCs before grafting for spinal cord injury contributes to repair of the injured spinal cord. In comparison with other reports on the transplantation of treated MSCs for spinal cord injury repair, the method we used with VHL peptide showed equal or greater functional recovery in the BBB locomotor scale [6,13].

In conclusion, neuronal lineage marker-positive cells are efficiently generated from MSCs with intracellular delivery of the TATVHL(157–171) peptide, and also that the engrafted TATVHL(157–171) peptide-delivered MSCs restore the physiological function in the spinal cord injury model rats. This study provides a promise of clinical application of autologous donor cells derived from readily accessible bone marrow for the cell-transplantation therapy for patients with spinal cord injury.

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REVIEW ARTICLE

Glioma-initiating cells and molecular pathology: implications for therapy

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Abstract There is now compelling evidence that gliomas harbor a small population of cells, termed glioma-initiating cells (GICs), characterized by their ability to undergo self-renewal and initiate tumorigenesis. The development of therapeutic strategies targeted toward GIC signaling may improve the treatment of malignant gliomas. The characterization of GICs provides a clue to elucidating histological heterogeneity and treatment failure. The role of the stem cell marker CD133 in the initiation and progression of brain tumors is still uncertain. Here, we review some of the signaling mechanisms involved in GIC biology, such as phosphatase and tensin homolog (PTEN), sonic hedgehog, Notch, and WNT signaling pathways, maternal embryonic leucine-zipper kinase (MELK), BMI1, and Janus kinase signal transducer and activator of transcription (JAK-STAT) signaling. In addition, we discuss the role of microRNAs in GICs by focusing on microRNA-21 regulation by type I interferon.

Keywords Glioma-initiating cells · CD133 · Molecular pathways · STAT3 · MicroRNAs

Introduction

Cancers are mostly composed of heterogeneous cell populations. A significant degree of morphological and lineage heterogeneity may contribute to tumor expansion, invasion, and metastasis, as well as drug resistance [1]. Glioblastoma multiforme (GBM) is the most lethal primary glioma, and patients diagnosed with this type of glioma typically have a median survival of less than 15 months, despite various therapies that include surgical resection, radiotherapy, and chemotherapy [2]. As the term "multiforme" suggests, the histopathology of this tumor is extremely variable. These extensively heterogeneous bulk tumors are composed of differentiated and undifferentiated cells and are often linked to a propensity for invasion and drug resistance [3]. Elucidating the mechanisms underlying tissue heterogeneity in GBM may be crucial for treating this disease. Such multiple distinct subpopulations of cancer cells within tumors may be derived from a limited source of cancer cells that have plasticity and respond to the signals they receive from their microenvironment [4]. Recent studies revealed that several types of tumors contain a minor population of tumor-initiating cells, called stem-like cancer cells, or cancer stem cells [5, 6]. These cells can aberrantly differentiate into diverse cell types in response to oncogenic cues. Such stem-like cancer cells have been well characterized in GBM and are referred to as glioma-initiating cells (GICs) [7–9]; thus, GICs may provide clues to elucidating the histological heterogeneity of GBM.

Glioma-initiating cells as a clue to elucidating heterogeneity and treatment failure

Nestin is an intermediate filament cytoskeletal protein found in neuroepithelial stem cells and progenitor cells [10]. Glial-derived neoplasms also express nestin, the expression level of which is higher in high-grade than in low-grade gliomas [11]. By using the neurosphere assay,

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Ignatova et al. [12] isolated nestin-expressing cells capable of forming clones that exhibit intraclonal heterogeneity for neural lineage-specific protein expression. Subsequently, Singh et al. cultured glioma cells in serum-free medium containing the stem cell growth factor's basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). They found that a small to moderate percentage of glioma cells (range 0.3–25.1%), depending on tumor aggressiveness, were capable of self-replication, formed nonadherent neurospheres, and were maintained in culture over time through multiple passages [13]. These self-renewing and tumor-culture-maintaining cells not only stained positive for the undifferentiated neural stem cell (NSC) marker nestin but also stained for CD133, a hematopoietic stem cell marker present on normal human NSCs. However, these cells did not express β-tubulin III or glial fibrillary acidic protein (GFAP), which can be used as markers for differentiated neural lineages. In stark contrast to this small group of CD133+ cells, the majority of glioma cells were found to be CD133− and incapable of forming self-sustaining neurospheres. As a general observation, a fraction of CD133+ GICs from aggressive tumors exhibited an increased rate of self-renewal relative to that from less aggressive tumors. Under culture conditions that promote differentiation, GICs lose the expression of primitive markers such as CD133 and nestin and instead express differentiation markers for the cell of origin. When these GICs were serially transplanted into the brain of a non-obese diabetic, severe combined immunodeficient (NOD-SCID) mouse, they produced exact phenocopies of the original tumor with all the histopathological features and cell-surface markers of the original tumor (Fig. 1). Immunohistological staining of these GBM xenografts revealed differential staining for CD133 and GFAP, underlining the fact that GICs can differentiate into mature progeny.

Even though the presence of GICs can clearly account for the inherent heterogeneous nature of gliomas, GIC cellular and genetic analyses showed that these cells are genetically transformed with enhanced self-renewal properties and possess an abnormal karyotype, which is not only limited to CD133+ cells but is also present in CD133− cells. This suggests that all cancer cells are derived from multiple clones. Even though a consensus has not yet been reached on the exact mechanism of gliomagenesis, the new-found understanding of the presence of a functional hierarchy in gliomas indicates that it will be important to investigate the slow-growing mutated GICs in order to characterize the mechanisms of treatment failure. GICs are resistant to a wide variety of chemotherapeutic agents and possess the remarkable ability to recover from cytotoxic therapy [14]. Kang et al. reported that a small population of multipotent CD133+ cancer cells can survive and proliferate upon exposure of GBM cells to a lethal dose of carmustine (BCNU). When these cells were transplanted into the brain of a SCID mouse, the original tumor was reproduced [15]. A significantly higher level of CD133+ cells was present in previously treated GBM than in newly diagnosed GBM [16]. The gene profile of CD133+ cells exhibits a high expression level of antiapoptotic genes and chemotherapy resistance genes, such as *breakpoint cluster region psuedogene 1 (BCRP1)* and *O^6-methylguanine-DNA-methyltransferase (MGMT)* [16], rendering these cells resistant to many commonly used chemotherapeutic agents, including temozolomide, carboplatin, paclitaxel (Taxol), and etoposide (VP16). In the same tumor, the expression of genes such as multidrug-resistance-associated proteins (*MDR*) 1 and 3 was markedly elevated in GICs when compared with levels in non-GICs [17], emphasizing their role in chemoresistance.

GICs play a crucial role also in radiation therapy failure, as tumors surviving radiotherapy are enriched in GICs. In a study conducted by Bao et al. [18], the irradiation of an in vivo glioma xenograft led to a three- to fivefold increase in the CD133+ cell population relative to untreated xenografts [18]. This suggests that irradiation leads to the enrichment of CD133+ cells in the tumor and the subsequent formation of more aggressive tumors with decreased latency after serial transplantation. Given the

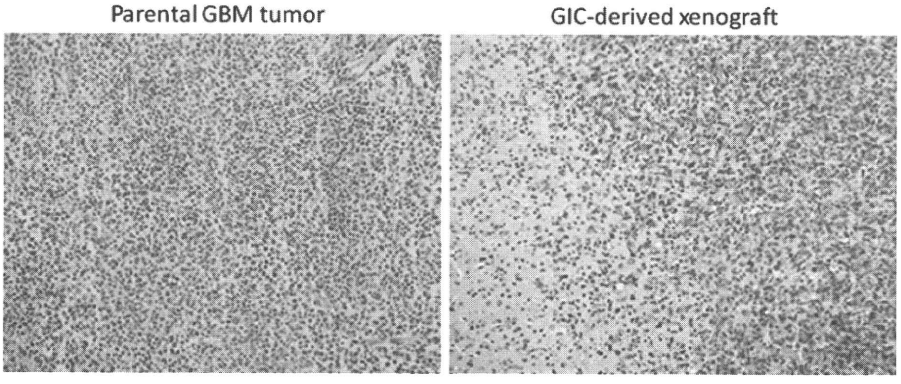

Fig. 1 Parental tumor and glioma-initiating cell (GIC)-derived intracranial xenograft tumor [hematoxylin and eosin (H&E) ×200). Our experimental data

pattern of treatment failure observed with current standard therapy, an alternative strategy involving selective targeting of this functionally distinct chemo- and radiation-resistant small group of GICs, instead of the bulk of the tumor, may provide more success in treating this deadly disease.

CD133: a definitive marker of glioma-initiating cells?

However, numerous reports suggest a less clear distinction between the ability of CD133+ and CD133− cells to form orthotopic tumors. The role of CD133+ cells versus other cell populations in the initiation and progression of brain tumors is still uncertain [19]. Within the C6 glioma cell line, even CD133− cells possess clonogenic, self-renewal, and tumorigenic capacities [20]. A recent study reported that CD133− cells isolated from primary GBM were equally as capable of forming brain tumors as the CD133+ subpopulation, whereas under the same conditions, none of the secondary GBM (0 of 7) produced viable neurosphere cultures. The study also reported that in four of 11 primary GBMs, CD133− cells grew as an adherent monolayer and yet were able to produce brain tumors [21]. Similarly, CD133− primary GBM cells, maintained as an adherent monolayer by the addition of serum to stem cell culture media, also produced highly infiltrative orthotopic tumors [22]. CD133− glioma cells are tumorigenic in nude rats, and CD133+ cells can be obtained from these tumors. Upon passaging of these tumors in vivo, CD133 expression is upregulated, coinciding with the onset of angiogenesis and reduced survival. Thus, the findings suggest that CD133 expression may not be required for brain tumor initiation, but CD133 expression coincides with the onset of tumor angiogenesis and reduced survival in a rat-brain tumor model [23]. Whereas many questions remain for GIC markers, potential of these markers to increase our understanding of tumor development and therapy design and selection is exciting indeed.

Hierarchy and histology derived from CD133+/− cells

Chen et al. observed that individual GBM can contain CD133+ and CD133− cell types that are capable of self-renewal, initiation of neurosphere growth, and generation of highly malignant tumors [24]. They hypothesized that GBM cells are classified into three clonogenic neurosphere cell types: type I cells are CD133− cells that generate cultures containing a mixture of CD133+ and CD133− cells; type II cells are CD133+ cells that also generate mixed cultures of CD133+ and CD133− cells; and type III cells are CD133− cells that only generate cultures of CD133− cells (Fig. 2). The authors stated that GBMs may consist of these three clonogenic cell types with lineage relationships between them. Under neurosphere culture conditions, clonogenic cultures of all cell types express neural stem cell markers, e.g., nestin, and they also contain a mixture of cells expressing several differentiation markers for neurons, astrocytes, and oligodendroglia, indicating that all three clonogenic cell types possess neural stem cell properties and the capability for multilineage differentiation.

Although all type I, II, and III cells are capable of generating grafts in nude mice, according to Chen et al. [24], these cell types have distinct growth kinetics, histological features, and molecular signatures. Grafts of type I CD133− self-renewing cells generate aggressive and faster-growing tumors, which show diffuse borders between tumor and brain tissue. Furthermore, type I grafts produced more elongated tumor cells than did type II and III grafts. In contrast, type III CD133− cells generate slow-growing and circumscribed lesions of CD133− cells; however, the proliferation indices of type III clone grafts are higher than those of type I grafts. Furthermore, type II CD133+ clone grafts have intermediate features between type I and III clone grafts. Consistent with the histological features of type I, II, and III cells, each clonal culture and graft revealed that type I and II cells express radial glial markers, including fatty-acid-binding protein (FABP) or Brain lipid binding protein (BLBP) and other neural stem cell markers such as nestin and Glial high affinity glutamate transporter (GLAST) at a much higher level than in type III cells. Of note, FABP has been previously linked to the invasiveness and poor prognosis associated with GBM [25, 26]. In particular, FABP7 expression within type I and II grafts was intense in elongated invading tumor cells. In contrast, type III cultures and grafts had lower FABP7 expression levels, whereas type III cell cultures expressed markers of intermediate progenitor cells, including T-box brain protein 2 (TBR2), distal-less homeobox 2 (DLX2), DLX1, and Cuticlin-like 2 (CUTL2). This indicates that type I cells represent the most primordial state, whereas type III cells represent the most differentiated state, and type II clones are intermediate between the type I and III clones.

Their proposed model demonstrate that CD133+ and CD133− cells are self-renewing tumor-initiating GBM cells and have the capability to generate serial transplantable tumors in nude mice. Tumors from patients may contain multiple distinct populations of lineally related CD133− cells representing different stages of differentiation. This finding should help explain the heterogeneity of the GBM phenotype. Although their study gives rise to a suitable explanation for the controversy regarding CD133+/− expression in GICs, it also has an important limitation. Their observations do not resolve the issue of the original GBM cell type, even though it revealed the relationship of the

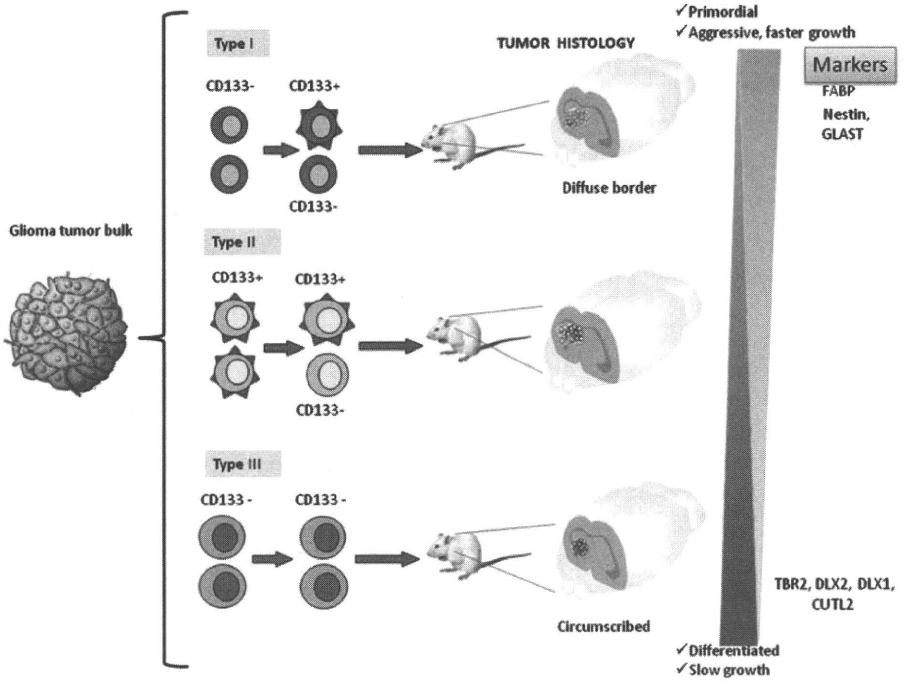

Fig. 2 According to Chen et al. [24], glioblastoma multiforme (GBM) cells are classified into three clonogenic neurosphere cell types that produce different patterns of cells. All cell types can generate grafts in nude mice, but each cell type has distinct growth kinetics, histological features, and molecular signatures. Grafts of type I CD133− cells generate aggressive and fast-growing tumors. Type I cells also express higher levels of radial glial markers, including FABP7, and neural stem cell markers such as nestin and GLAST. Type III CD133− cells produce slow-growing and circumscribed lesions of CD133− cells. Type III cells express markers of intermediate progenitors, such as TBR2, DLX2, DLX1, and CUTL2. Type II CD133+ clone grafts show intermediate features between type I and III clone grafts

lineage of self-renewing tumor cell capabilities pertaining to the GBM phenotype. In addition, the orthotropic neurosphere graft in their study could reconstitute tumors, but with a lack of microvascular proliferation and a mesenchymal structure. Further studies are required for a thorough understanding of the role of different self-renewing cell types in microenvironment formation as well as tumor cell adaptation to the microenvironment.

Signaling pathways of glioma-initiating cells

Several signaling pathways that regulate normal stem cell self-renewal cause neoplastic proliferation when dysregulated by mutations. Emerging evidence has addressed the roles of the phosphatase and tensin homolog (PTEN) [27, 28], sonic hedgehog (SHH) [29, 30], Notch [31, 32], Wingless-type MMTV integration site family member (WNT) [33, 34], maternal embryonic leucine-zipper kinase (MELK) [35], and B lymphoma mo-MLV insertion region 1 (BMI1) [36, 37] pathways of stem cell self-renewal with neoplastic proliferation (Table 1).

PTEN suppresses Akt phosphorylation by reversing PI3 kinase (PI3K)-driven phosphorylation. *PTEN* is a suppressor gene that is frequently deleted in GBM. *PTEN* deletion, in cooperation with *TP53* loss, enhances self-renewal, proliferation, and tumorigenic potential in NSCs [38]. *PTEN* deletion with retinoblastoma associated protein (pRb) inactivation or ABCG2 transporter activation accelerates the formation of aggressive high-grade tumors and GIC-like neurosphere formation capacity in a transgenic mouse model of glioma [28, 39–42]. The role of *PTEN* status in GIC still needs to be elucidated, and this molecule is one of the most remarkable targets involved in GIC activity.

The WNT and SHH signaling cascades promote proliferation of numerous cells, including granule-cell progenitors of the cerebellum, the presumed origin cells of medulloblastomas. WNT signals are divided into two different pathways: the canonical, or WNT/β-catenin, pathway is involved in cell-fate determination, whereas the noncanonical pathway is involved in the control of cell movement and tissue polarity [43]. Epigenetic silencing and loss-of-function mutations of negative regulators of WNT signaling are observed in a variety of human cancers. In sporadic medulloblastoma, the mutation of several downstream genes of WNT/β-catenin signaling, including Axin, β-catenin, and Adenomatosis polyposis coli (APC) and constitutive WNT signaling activation were found [44, 45].

Table 1 Signaling pathways in glioma-initiating cells

Signaling pathway	Downstream molecules	Normal biology	Biology and effect in GBM and GICs
PTEN	PIP3 (Akt Pathway), p53	Suppresses Akt phosphorylation, suppress p53 degradation	Frequently deleted with TP53 loss in GBM, pRb inactivation, ABCG2 transporter activation; accelerates the formation of aggressive high-grade tumors; enhances GIC-like neurosphere formation capacity
WNT	Axin, β-catenin, APC	Cell fate determination (canonical pathway); controls cell movement and tissue polarity (noncanonical)	Constitutive WNT signaling activation
SHH	PTCH, SMO, GLI	Maintains cell stemness, survival, and proliferation	Regulates Stemness genes: *NANOG*, *SOX2* and *OCT4*; promotes growth, survival, and proliferation of GBM and GICs
Notch	HES1, phospho-S6RP, GLUT1	Maintains neural-cell stemness, survival, and proliferation; controls specification, proliferation, and survival of neural precursors	Enhances DNA damage response via activation of PI3K/Akt pathway; promotes cell growth chronogenic survival, tumor formation of GBM
MELK		Enhances cell survival under condition of nutrient starvation; controls self-renewal of neural stem cell	Regulates proliferation, survival, and apoptosis of GICs; MELK expression is negatively correlated with poor GBM prognosis
BMI1	p14INKa, p16ARF	Prevents neural stem cell senescence, apoptosis, and differentiation	Highly expressed in GBM and GICs; inhibits apoptosis; attenuates GIC differentiation; inhibits activation of tumor suppressor pathways

PTEN phosphatase and tensin homolog, *SHH* sonic hedgehog, *MELK* maternal embryonic leucine-zipper kinase, *HES1* hairy and enhancer of split 1, (Drosophila), *GBM* glioblastoma multiforme, *GICs* glioma-initiating cells, *APC* adenomatous polyposis coli, *PTCH* patched homolog (Drosophila), *SMO* smoothened homolog (Drosophila), *GLI* gliotactin, *GLUT1* glucose transporter 1, *NANOG* nanog homebox, *SOX2* sex determining region Y-box 2, *HES1* hairy and enhancer of split 1, *Phosho-S6RP* phosho-S6 ribosome protein

SHH is a secreted protein critical for pattern formation of the central nervous system [46]. SHH ligand binding to its receptors, patched homolog (PTCH) and smoothened homolog (SMO), leads to the activation of gliotactin (GLI) transcription factors that are translocated into the nuclease to regulate various cellular activities, including maintenance of cell stemness, survival, and proliferation. Although the involvement of SHH in tumorigenesis is best characterized in medulloblastoma, an increasing body of evidence indicates the involvement of SHH in GBM tumorigenesis.

PTCH expression colocalizes with stem-like cells expressing Ki67 and BMI1, but not with GFAP, which is considered as a differentiation marker of NSCs [47]. Altaba et al. also demonstrated that SHH signaling regulates the expression of stemness genes such as *nanog homeobox (NANOG)*, *SRY-box containing gene 2 (SOX2)*, and *Octamer binding protein 4 (OCT4)*. According to their study, SHH-GLI signaling is required not only for sustained glioma growth and survival but also for GIC survival and proliferation. Interference of SHH-GLI signaling with cyclopamine, a steroid alkaloid of SMO, drastically attenuated the self-renewal, proliferation, and increased apoptosis of GICs. In addition, cyclopamine induces additive and synergistic effects with temozolomide, the most commonly used chemotherapeutic agent for GBM. This finding reveals the essential role of SHH signaling in regulating the behavior of GICs and offers new therapeutic possibilities for GBM [48].

Notch controls specification, proliferation, and survival of nonneoplastic neural precursors and is aberrantly activated in embryonal brain tumors, suggesting a molecular link between NSCs and medulloblastoma. Notch blockade suppresses the expression of the pathway target Hes1 and causes cell-cycle exit, apoptosis, and differentiation in medulloblastoma cell lines. Previously, Sullenger et al. demonstrated that GICs promote radioresistance compared with the GBM tumor bulk because GICs preferentially activate the DNA damage-response pathway. Notch signaling plays an important role in this DNA damage-response pathway via the activation of the PI3K/Akt pathway and the prosurvival protein Mcl-1. Notch pathway inhibition using γ-secretase inhibitors impairs cell growth, clonogenic survival, and tumor formation ability and sensitizes GICs to radiation at clinically relevant doses [49].

MELK encodes a serine/threonine kinase with a leucine zipper domain and is a poorly characterized member of the sucrose non-fermenting protein kinase 1/adenosine monophosphate-activated protein kinase (SNF1/AMPK) family. Several members of this family are known as tumor survival factors under conditions of nutrient starvation. MELK is highly expressed in normal NSCs and glioma-initiating/tumor progenitor cells and plays a role in regulating their

proliferation, survival, and apoptosis [50]. As the MELK expression is negatively correlated with the progress of GBM patients, MELK could be a novel target to reveal the role of GICs in GBM malignancy.

BMI1 binds to p14INKa and p16ARF and inhibits activation of their signaling. These two downstream genes are highly expressed in primary GBM and oligodendrocytoma, another type of glioma. Several studies have demonstrated that elevated expression of BMI1 was detected in tumor-derived neurospheres, suggesting that BMI1 plays an important role in GIC proliferation [35, 51]. Other studies by Abdouh et al. demonstrated that the stable knockdown of BMI1 inhibited clonogenic potential in brain tumor formation in vitro and in vivo. BMI1 prevented apoptosis of tumor-derived progenitor cells expressing CD133+ and attenuated the differentiation of neurons and astrocytes. Furthermore, gene-expression analysis suggested that BMI1 inhibits activation of tumor suppressor pathways that drive *INK4A/ARF/TP53* deletion and PI3K/AKT hyperactivity [52].

STAT3 activation in glioma-initiating cells

Signal transducer and activator of transcription 3 (STAT3) activation is crucial for stem cell function, differentiation, and survival. Bone morphogenetic protein (BMP) and leukemia inhibitory factor/ciliary neurotrophic factor (LIF/CNTF) induced the STAT3-mediated differentiation of NSCs and GICs [8]. The study indicates the potential for therapeutic approaches that can induce GICs to undergo terminal differentiation. We previously demonstrated that STAT3 expression is strongly upregulated in GICs, and that interferon (IFN)-β phosphorylates a tyrosine residue of STAT3. IFN-β treatment elicits a remarkable antiproliferative effect and enhances the expression of oligodendroglial markers, including oligodendrocyte-specific protein (OSP), galactosylceramidase (GalC), and myelin-basic protein (MBP), by GICs. However, the expression of these markers was inhibited by a peptide that specifically inhibits STAT3 (Fig. 3). Furthermore, IFN-β treatment did not induce the expression of OSP, GalC, and MBP in human NSCs. In contrast, whereas the expression of other neural lineage markers (e.g., GFAP and neuron-specific class III β-tubulin) was unaffected, the expression of nestin and CD133 was reduced in GICs after IFN-β treatment. IFN-β-treated GICs were unable to form tumors in NOD-SCID mice [53]; therefore, IFN-β may represent a potential therapeutic agent for inducing the terminal differentiation of GICs.

MicroRNA in glioma and glioma-initiating cells

MicroRNAs (miRNAs) are small, noncoding RNAs consisting of 20–22 nucleotides that participate in the posttranslational regulation of gene expression by means of RNA

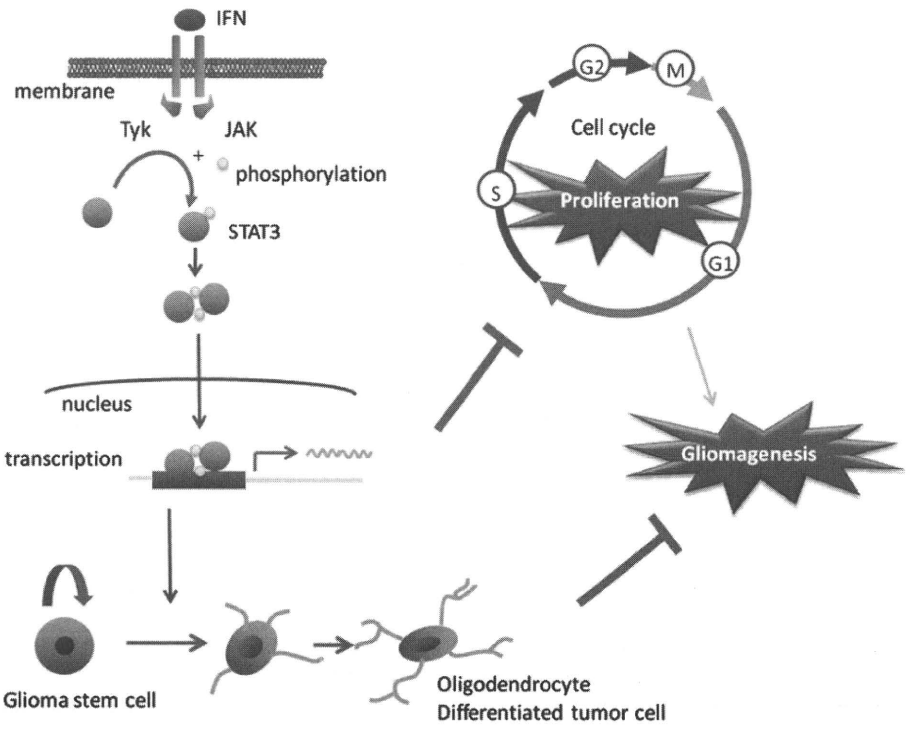

Fig. 3 Interferon (IFN)-β phosphorylates a tyrosine residue of signal transducer and activator of transcription 3 (STAT3). IFN-β treatment elicits a remarkable antiproliferative effect. IFN-β induces glioma stem cells to undergo terminal differentiation to mature oligodendrocytes that express oligodendrocyte-specific protein (OSP), galactosylceramidase (GalC), and myelin basic protein (MBP), and inhibits gliomagenesis. This is a schema based on the report by Yuki et al. [53]

interference (RNAi). The miRNA genes are transcribed by RNA polymerase II in the nucleus to form large primary-miRNA (pri-miRNA) transcripts. These pri-miRNA transcripts are processed by the Drosha enzyme to release the pre-miRNA precursor product, which is <70 nucleotides in length. After the precursor-miRNA (pre-miRNA) is transported into the cytoplasm, another enzyme known as Dicer processes the intermediate molecule to generate a mature 22-nucleotide miRNA. This mature miRNA is integrated into the RNA-induced silencing complex (RISC) and forms double-stranded RNA (dsRNA) with complementary messenger RNAs (mRNAs). Depending on the degree of homology between the miRNA and the mRNA, RISC can inhibit the function of mRNA by either promoting its cleavage or inhibiting its translation [54]. Emerging evidence suggests that miRNAs are involved in crucial biological processes, including the development, differentiation, apoptosis, and proliferation of mammalian cells. In humans, miRNAs have been proposed to contribute to oncogenesis because they possess multifaceted functions either as tumor suppressors or as oncogenes [55]. Although the complete understanding of miRNA biology in gliomas and GICs still requires a tremendous amount of study, several miRNAs have been reported to be involved in gliomas and GICs (Table 2).

MiRNA-124 (miR-124) and miR-137 are significantly downregulated in GICs relative to nonneoplastic brain tissue. The overexpression of miR-124 and miR-137 induces morphological changes and expression of differentiation markers in GICs, indicating that they induce the differentiation of GICs [56]. MiR-128 expression is also significantly decreased in glioma cells [57]. MiR-128 transfection into glioma cells caused a remarkable reduction in their proliferation in vitro and in vivo, and its overexpression in NSCs and GICs significantly decreased the volume of neurospheres following the loss of BMI1. These studies suggest that miR-128 may control stem-cell renewal in NSCs and GICs. MiR-326 is weakly expressed in glioma cells and GICs. Kefas et al. revealed that miR-326 acts in a negative feedback loop with Notch signaling. The re-expression of miR-326 in glioma cell lines and GICs leads to their decreased viability, proliferation, and invasiveness by the inhibition of Notch. Kefas et al.'s study demonstrated an miR-326/Notch axis, and this axis could shed light on the biology of Notch and miR-326 in GICs [58]. Ernst et al. [59] demonstrated that the miR-17-92 cluster consists of several miRNAs amplified and overexpressed in GICs, such as miR-17-3p, miR-17-5p, and miR-92a-1. In contrast, expression of the miR-17-92 cluster is downregulated upon differentiation induction of the GBM spheroid. The authors also revealed that miR-17-92 functions as a negative regulator of cyclin-dependent kinase inhibitor 1A (CDKN1A), E2F1, and PTEN expression in GICs, and its potent target is connective tissue growth factor (CTGF). Inhibition of the miR17-92 cluster leads to increased apoptosis and decreased GIC-cell proliferation. The results of that study suggest that interaction between miR-17-92 and its target CTGF may be critical for differentiation-promoting treatment of glioma cells. MiR-21 is one of the most commonly upregulated miRNAs in glioma, and it also modulates tumorigenesis through regulation of

Table 2 Role of microRNAs (miRs) in glioma-initiating cells

MicroRNA	Normal biology	Expression in glioma	Expression in glioma biology and function in glioma and GICs	Targets
miR-124	Promotes neural differentiation expressed in differentiating and mature neurons	Downregulated	Overexpression slows proliferation promotes neural differentiation	*ITGB1* *LAMC1* *CDK6* *SCP1* *PTBP1*
miR-137	Not known	Downregulated	Same as miR-124	*CDK6*
miR-128	Not known	Downregulated	Overexpression slows proliferation blocks GICs self-renewal	*BMI1*
miR-326	Negative feedback loop with Notch	Downregulated	Overexpression inhibits Notch decreases viability, proliferation, and Invasion	*Notch*
miR-17-92 cluster	Promotes neural differentiation	Upregulated	Regulates CDKN1A, E2F1, and PTEN, repression increases apoptosis of GICs decreases proliferation of GICs	*CTGF*
miR-21	Antiapoptotic factor	Upregulated	Repression inhibits tumor growth; increases apoptosis; induces cell-cycle arrest	*p53 TGF-beta APAF1 bcl2PTEN tropomyosin-1 PDCD4*

PTEN phosphatase and tensin homolog, *GBM* glioblastoma multiforme, *GICs* glioma-initiating cells, *CDKN1A* cyclin-dependent kinase inhibitor 1A, *ITGB1* Integrin beta 1, *LAMC1* lamin, gamma 1, *SCP1* Small C-terminal domain phosphatase 1, *PTBP1* Polypyrimidine tract binding protein 1, *CDK6* Cyclin-dependence kinase 6, *APAF1* Apoptotic peptidase activating factor 1, *PDCD4* Programmed cell death 4

genes such as TP53, transforming growth factor (TGF)-beta, APAF1, bcl-2, PTEN, tropomyosin-1 (TPM1), and PDCD4 [60–62]. Previously, miR-21 was suggested to be aberrantly expressed and was recognized as one of the major antiapoptotic factors in malignant gliomas [63]. MiR-21 inhibition led to repressed growth, increased apoptosis, and cell-cycle arrest by de-repressing its target genes. Although the function of miR-21 in stem cell biology remains controversial, it may target the known stem cell regulators NANOG and SOX2 that are critical for stem cell self-renewal [64].

Regulation of microRNAs by STAT3 in glioma-initiating cells

RNAi induces a multitude of responses in addition to gene knockdown. This is best understood in the context of the antiviral immune response. In particular, dsRNA, a nucleic acid associated with viral replication, is involved in numerous interactions contributing to induction, activation, and regulation of antiviral mechanisms. One particularly intriguing function of dsRNA is its stimulation of important protective responses, such as the activation of Dicer-related antiviral pathways, induction of type I IFN (IFN-α/β), and stimulation of dsRNA-activated protein kinase and oligoadenylate synthase [65]. IFN-α/β regulates the levels of crucial mediators of the antiviral response, such as protein kinase R, the 2′–5′ oligoadenylate synthase/RNase L system, adenosine deaminase ADR1, and Mx guanosine triphosphatase (GTPase) [66]; thus, RNAi may be involved in IFN-mediated antiviral response. It was recently reported that the levels of a liver-specific miRNA, miR-122, and several other miRNAs are regulated by IFN-β in human hepatoma cells and that IFN-β rapidly modulates miRNA expression that targets the genomic RNA of the hepatitis C virus, thereby inhibiting viral replication [67]. In addition to its ability to interfere with viral replication, IFN-β is also known for its antiproliferative effects in a variety of neoplasms, such as hepatocellular carcinoma, chronic myeloblastic leukemia, melanoma, renal cancer, and glioma [68]. Therefore, IFN-β may induce or downregulate cellular miRNAs in human neoplasms and use the RNAi system in its action against tumor progression.

We tested whether IFN-β can alter cellular miRNA expression in human glioma cells by using data obtained from a genome-wide microarray [69]. On the basis of the initial screening, we found that expression levels of several miRNAs were increased (miR-187 and miR-194) or attenuated (miR-100, miR-21, and let-7 family miRNAs) in response to IFN-β treatment. We focused on miR-21 because it is one of the best-known miRNAs associated with tumorigenesis and progression in gliomas, as described above. We demonstrated that miR-21 overexpression occurs in a surgical specimen of glioblastoma by performing in situ hybridization analyses (Fig. 4a–c). We also compared miR-21 expression levels in glioma cell lines, GICs, and normal brain tissue. MiR-21 was overexpressed in glioma cells relative to normal brain cells. Notably, miR-21 expression level was higher in GICs than in the glioma cell lines (Fig. 4d). This finding may indicate that miR-21 plays a crucial role in the initiation and progression of glioma.

Investigations using quantitative real-time-polymerase chain reaction (qRT-PCR) revealed that IFN-β downregulates miR-21 in cultured glioma cell lines and GICs, and that the systemic delivery of IFN-β reduces the level of miR-21 in intracranial GIC xenografts in mice. In time-course experiments, IFN-β treatment showed a relatively fast response in reducing miR-21 levels, suggesting that the negative regulation of miR-21 may be mediated directly by IFN-β, for example, through phosphorylation of Janus kinase/STAT (JAK/STAT). Our finding that IFN-β also suppresses pri-miR-21 and pre-miR-21 expression suggests that it regulates miR-21 transcription. The putative regulatory region of the *miR-21* gene is located within an intron of the overlapping transmembrane protein 49 gene (*TMEM49*). This regulatory region contains two consensus STAT3-binding sites located approximately 800 bp upstream from the transcription start site. We used a luciferase reporter system with a fused full-length pri-miR-21 promoter, including the STAT3 binding site, to demonstrate that IFN-β inactivates STAT3-mediated miR-21 promoter activity and that transcriptional activity could be restored by using a STAT3 inhibitor. These data suggest that miR-21 expression is negatively regulated by STAT3 activation induced by IFN-β.

In conclusion, the downregulation of miR-21 in response to IFN-β treatment contributes to the antitumor effects of this cytokine in GICs; furthermore, miR-21 expression is negatively regulated by STAT3 activation (Fig. 5). These results highlight the importance of understanding the transcriptional regulation of miRNAs that are involved in the oncogenesis of gliomas.

Targets for future brain tumor therapy

GIC resistance to chemotherapy and radiotherapy could be targeted with inhibitors. Molecular analysis of the GIC population may lead to the identification of novel pathways important for their proliferation, self-renewal, and differentiation, thus opening up new targets for therapy. It will likely be important to study the effect of oncogene activation or tumor suppressor gene inactivation in stem cells as opposed to other brain cells, as these alterations are

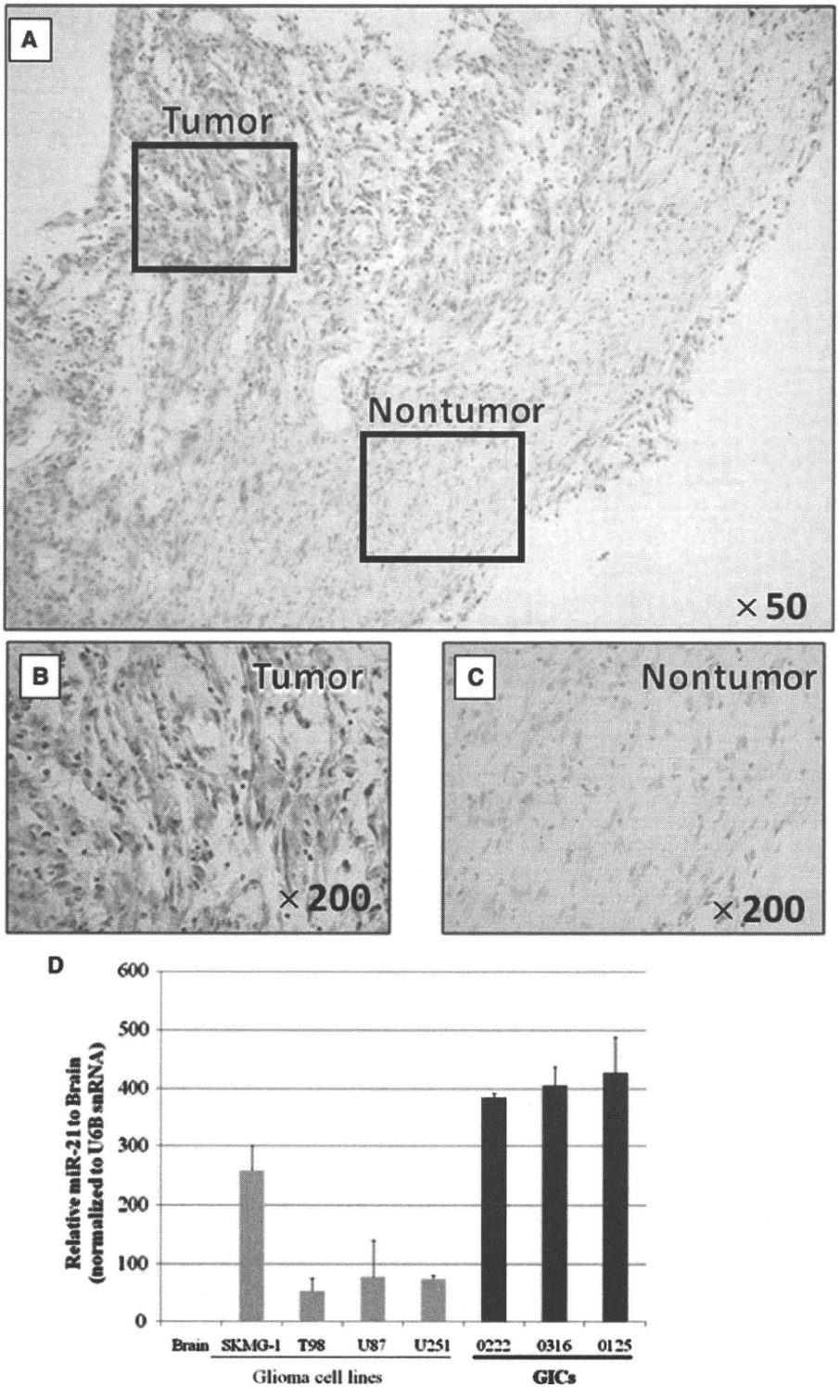

Fig. 4 MicroRNA-21 (miR-21) overexpression in glioma-initiating cells (GICs). An miR-21-specific probe was hybridized in situ with glioblastoma tissue obtained from a surgical specimen. The probe clearly stains glioblastoma tissue, but does not stain normal cortex tissue (**a**). Tumor cells express significant amounts of miR-21, as observed under high magnification (**b**), whereas nontumorous tissue does not (**c**). Quantitative real-time polymerase chain reaction (qRT-PCR) shows that miR-21 is overexpressed to a greater extent in glioma cells than in normal brain cells (**d**). Notably, the amount of miR-21 is greater in GICs than in glioma cell lines. *Columns* mean, *bars* standard deviation (normal brain expressed as 1). Modified from [69]

predicted to have different effects in stem cells versus differentiated cells. Since the identification of GICs, these cells have become promising targets for the treatment of brain tumors. It has also been considered for some time that an NSC or progenitor cell that resides in the brain may be a target for transformation, leading to a brain tumor. The potential that an NSC may be transformed into a brain tumor has been considered on the basis of the observations

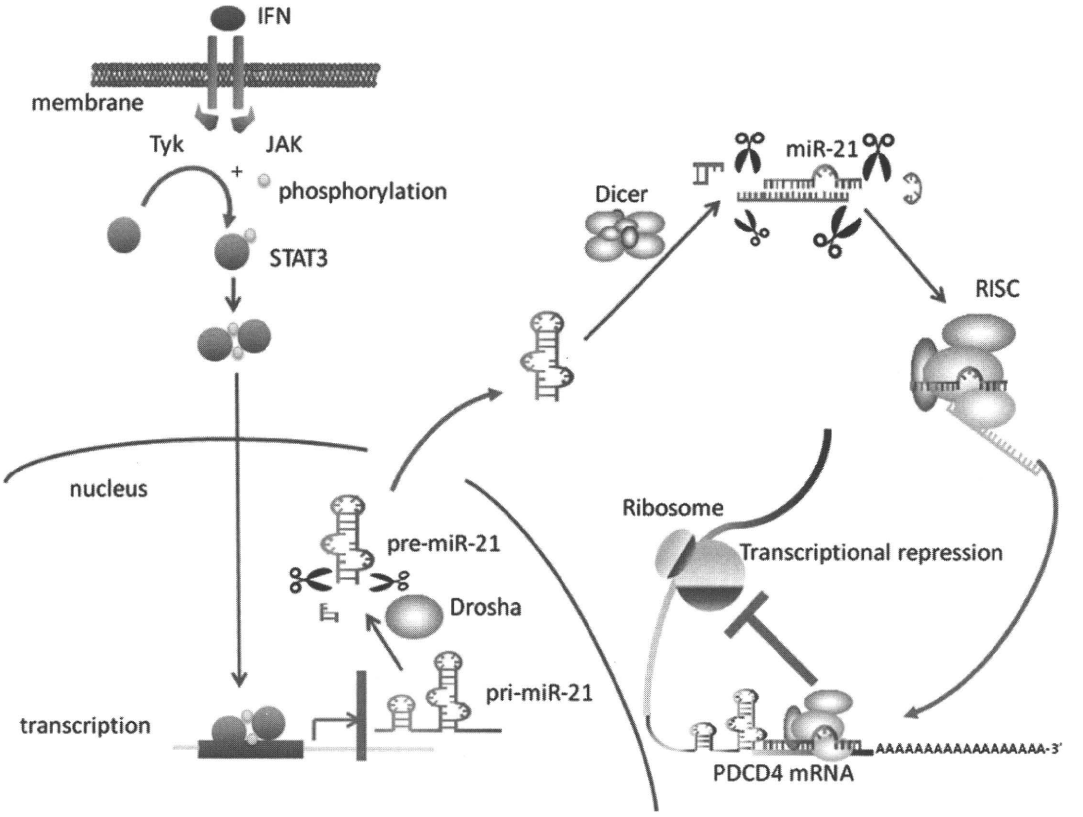

Fig. 5 Interferon (IFN)-β inactivates signal transducer and activator of transcription 3 (STAT3)-mediated microRNA-21 (miR-21) promoter activity. The *miRNA* genes are transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. These pri-miRNA transcripts are processed by the Drosha enzyme to release the pre-miRNA precursor product, which is <70 nucleotides in length. After the pre-miRNA is transported into the cytoplasm, another enzyme known as Dicer processes the intermediate molecule to generate a mature 22-nucleotide miRNA. This mature miRNA is integrated into the RNA-induced silencing complex and forms double-stranded RNA with complementary messenger RNAs of genes such as *bcl-2*, phosphatase and tensin homolog (*PTEN*), tropomyosin-1, and *PDCD4*. The schema is based on the article by Ohno et al. [69]

of tumors occurring in the brain's putative stem cell or proliferative zones in a number of older experimental systems. Several further lines of evidence suggest that brain tumors arise from the transformation of a normal NSC or progenitor cell, all of which rely on the recognition of the many functional and genetic similarities shared by somatic stem cells and cancer cells [70]. Thus, it is a problem imposed on us to identify a particular antigen or pathway of brain tumor stem cells.

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Original Article

Benefits of Interferon-β and Temozolomide Combination Therapy for Newly Diagnosed Primary Glioblastoma With the Unmethylated MGMT Promoter

A Multicenter Study

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BACKGROUND: The aim of the current study was to catalog genomic and epigenomic abnormalities in newly diagnosed glioblastoma patients and determine the correlation among clinical, genetic, and epigenetic profiles and clinical outcome. **METHODS:** This study retrospectively included 68 consecutive patients who underwent surgical treatment and received standard radiotherapy with temozolomide (TMZ)-based chemotherapy. Of a total of 68 patients, 39 patients (57.4%) received interferon (IFN)-β in combination of TMZ. **RESULTS:** The genetic and epigenetic alterations frequently observed were *EGFR* amplification (51.5%), *TP53* mutation (33.8%), *CDKN2A* loss (32.4%), *TP53* loss (16.2%), methylation of the MGMT promoter (33.8%) and *IDH1* mutation (5.9%). Multivariate analysis revealed that methylated MGMT promoter and the combination of TMZ and IFN-β were independent prognostic factors associated with survival. The median survival time (MST) of the patients who received the combination of IFN-β and TMZ was significantly greater with 19.9 months as compared to the TMZ alone group (12.7 months). Notably, in even patients whose tumors had unmethylated MGMT promoter, the MST prolonged to 17.2 months when receiving TMZ with IFN-β, compared to 12.5 months in those receiving TMZ without IFN-β. **CONCLUSIONS:** Taken together, addition of IFN-β for newly diagnosed primary GBM achieved a favorable outcome, particularly in patients with unmethylated MGMT promoter. ***Cancer* 2010;000:000–000.** © *2010 American Cancer Society.*

KEYWORDS: IDH1, MGMT methylation, glioblastoma, interferon-β, temozolomide.

Glioblastoma multiforme (GBM) is one of the most frequent primary brain tumors in the central nervous system in adults and is highly malignant, with a median survival time of about one year from diagnosis. This is despite aggressive treatment, surgery, postoperative radiotherapy, and adjuvant chemotherapy. An international randomized trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) comparing radiotherapy alone and concomitant radiotherapy and temozolomide (TMZ) clearly attested the benefits of adjuvant TMZ chemotherapy for GBM patients.[1] Since then, TMZ has been the current first-line chemotherapeutic agent for GBM.

A subanalysis in this trial showed the effectiveness of epigenetic silencing of the MGMT gene by promoter methylation with longer survival in patients with primary GBM; it also suggested the benefits of combining chemotherapy using TMZ with radiotherapy.[2]

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Furthermore, there have been recent attempts to comprehensively profile GBM genes by The Cancer Genome Atlas (TCGA) project and other groups.[3,4] Some genetic aberrations in GBM, such as *TP53* mutation or deletion, *NF1* deletion or mutation, and *ERBB2* mutation, have been found to be more common than previously reported. In addition, novel molecular markers, such as frequent mutations of the *IDH1* and *IDH2* genes in secondary GBM have been discovered.[5-7] These findings on mutations, genomic and epigenomic aberrations, and transcriptomal features in GBM might aid in understanding the classification of GBM and its further potential clinical implications.

However, the TCGA project included GBM patients who received surgical treatment, and detailed information on adjuvant chemoradiotherapy was not provided. Therefore, the close relationship between the gene profile provided by TCGA and chemotherapy regimens remains unknown.[3]

In this current study, we aimed to determine the correlation between clinical, genetic, and epigenetic profiles, and clinical outcome in newly diagnosed GBM patients who received TMZ-based chemotherapy. Interestingly, we found a significant beneficial outcome in patients receiving TMZ in addition to IFN-β. Moreover, our study discovered that GBM patients with the ummethylated O^6-methylguanine-DNA methyltransferase (MGMT) promoter, in particular, showed benefits from IFN-β.

MATERIALS AND METHODS

Patient population

We retrospectively reviewed 68 consecutive patients with newly diagnosed primary GBM who underwent surgical treatment at several academic tertiary-care neurosurgical institutions: Nagoya University Hospital, Hamamatsu University Hospital, Oita University Hospital, and Shizuoka Cancer Center from May 2006 through June 2010 after TMZ was approved as the treatment agent for malignant gliomas by the National Ministry of Health and Welfare of Japan. The diagnosis of GBM was established by histological confirmation according to the WHO guidelines[8,9] independently by at least two expert neuropathologists. The clinical, operative, and hospital course records were reviewed. Information collected from clinical notes included patient demographics, pre- and postoperative neuroimaging, and adjuvant therapy. Preoperative Eastern Cooperative Oncology Group performance status (ECOG PS) scores were assigned by the clinician at the time of evaluation and were available in the chart for review for all patients. The study was approved by the institutional review board at each participating hospital and complied with all provisions of the Declaration of Helsinki.

Treatment

Radiotherapy

After undergoing surgery, the patients received focal external-beam radiotherapy by conventional radiation planning to approximately 60 Gray (Gy) (±5% total dose), with daily concurrent TMZ at 75 mg/m^2 throughout the course of radiotherapy.

Chemotherapy

All patients received the standard Stupp regimen.[1] In the absence of grade 3 or 4 hematological excessive toxicity, TMZ administration was continued until clinical or radiological evidence of disease progression was observed. Of these 68 patients, 39 patients (57.4%) received adjuvant IFN-β treatment (Table 1). Patients in Nagoya University and Oita University received chemotherapy consisting of IFN-β. There were no significant differences in any of the clinical parameters and genetic, epigenetic parameters (i.e., age, sex, preoperative PS, tumor location, extent of resection, genetic and epigenetic alterations between the institutions using regimen with and without IFN-β. The IFN-β chemotherapy regimen comprised 3 million international units (MIU)/body administered intravenously on alternate days during radiotherapy and TMZ-induction chemotherapy.[10,11] At the end of the induction period, after a 4-week interval, the patients were administered 3 MIU/body of IFN-β on the first morning every 4 weeks during TMZ maintenance chemotherapy. In the case of tumor progression, salvage or second-line therapy was administered at the investigators' discretion; most patients received additional chemotherapy.

Response Evaluation During Treatment

Both radiological and clinical findings were used to evaluate the response. Follow-up magnetic resonance imaging (MRI) was performed for alternate cycles. If the MRI showed continued increase in enhancement, the case was considered as tumor progression. If re-resection was performed for a recurrent mass lesion, histological interpretation formed the basis for definitive diagnosis (treatment-related necrosis vs recurrent tumor).

Table 1. Clinical Characteristics[a]{TC}

Parameter	No. of Patients	%
	n=68	
Age(y)		
Median	55.0	
Range	12-84	
<40	12	17.6
≥40, <60	24	35.3
≥60	32	47.1
Sex		
Male	41	60.3
Female	27	39.7
Preoperative ECOG performance status		
Median	1	
Range	0-3	
Preoperative ECOG performance status		
≤1	45	66.2
>1	23	33.8
Tumor location		
Superficial	50	73.5
Deep	18	26.5
Surgery		
GTR	24	35.3
Non-GTR	44	64.7
Chemotherapy		
TMZ only	29	42.6
TMZ+ IFN-β	39	57.4

ECOG indicates Eastern Cooperative Oncology Group; PS, performance status; GTR, macroscopic (gross) total removal; TMZ, temozolomide.

Tumor samples and DNA Extraction

All patients provided their written informed consent for molecular studies of their tumor, and the protocol was approved by the ethics committee at each center. Sixty-eight brain tumor specimens were obtained at the time of first surgical resection.

Tumor tissue samples were immediately frozen and stored at −80°C until the extraction of genomic DNA. DNA was prepared using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Placental DNA was used as the normal control. The amount of DNA obtained from the tumor was sufficient for the subsequent genomic and epigenomic analyses.

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) was used for the determination of allelic losses and gains of the gene in the tumor samples. The analysis was performed using the SALSA MLPA KIT P088-B1 and P105-C1 in accordance with the manufacturer's protocol (MRC Holland, Amsterdam, Netherland).[12-15] Information regarding the probe sequences and ligation sites can be found at www.mlpa.com. Amplification products were separated on an ABI® 3130 × I Genetic Analyzer (Applied Biosystems, Foster City, CA) and quantified with Genemapper 4.0 software (Applied Biosystems). Duplicate experiments were performed to obtain accurate MLPA values. Data analysis was performed with an original Excel-based program based on MRC-Holland's procedures. Normalization for sample data was first performed on control probes, and each tumor sample was then normalized using the data on 2 control samples, using peripheral blood DNA. Single regression for control and tumor data slope correction was performed. Abnormal/normal ratio limits were set at 0.65 and 1.3. Statistical analysis was performed using the same Coffalyser software.

Pyrosequencing

Tumor DNA was modified with bisulfate using the EpiTect bisulfite kit (Qiagen, Courtaboeuf Cedex, France). Pyrosequencing technology was used to determine the methylation status of the CpG island region of MGMT as described previously.[16,17] We used the touchdown PCR method. The primer sequences used were the MGMT forward primer, 5′-TTGGTAAATTAAGGTATAGAGTTTT-3′, and the MGMT biotinylated reverse primer, 5′-AAA CAATCTACGCATCCT-3′. PCR included a denaturation step at 95°C for 30 s, followed by annealing at various temperatures for 45 s, and extension at 72°C for 45 s. After PCR, the biotinylated PCR product was purified as recommended by the manufacturer. In brief, the PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, and denatured using 0.2 N NaOH solution and washed again. Next, 0.3 mM pyrosequencing primer was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Westborough, MA). The pyrosequencing primer was 5′-GGAAGTTGGGAAGG-3′. Methylation quantification was performed using the provided software.

TP53 and IDH1/IDH2 Sequencing

Direct sequencing of the *TP53* exons 5 to 8 and *IDH1/IDH2* was performed as previously described.[7,18,19] The primer sequences are listed in Table 2.

Table 2. List of Primer Sequences for Direct DNA Sequencing{TC}

Gene name	Exon		Sequence
TP53	Exon 5	F	5′-TTATCTGTTCACTTGTGCCC-3′
		R	5′-ACCCTGGGCAACCAGCCCTG-3′
	Exon 6	F	5′-ACGACAGGGCTGGTTGCCCA-3′,
		R	5′-CTCCCAGAGACCCCAGTTGC-3′
	Exon 7	F	5′-GGCCTCATCTTGGGCCTGTG-3′
		R	5′-CAGTGTGCAGGGTGGCAAGT-3′
	Exon 8	F	5′-CTGCCTCTTGCTTCTCTTTT-3′
		R	5′-TCTCCTCCACCGCTTCTTGT-3′.
IDH1		F	5′-CGGTCTTCAGAGAAGCCATT-3′
		R	5′-GCAAAATCACATTATTGCCAAC-3′
IDH2		F	5′-AGCCCATCATCTGCAAAAAC-3′
		R	5′-CTAGGCGAGGAGCTCCAGT-3′.

F indicates forward primer; R, reverse primer;.

For IDH sequencing, a fragment 129 bp in length, spanning the sequence encoding the catalytic domain of *IDH1*, including codon 132, and a fragment 150 bp in length spanning the sequence encoding the catalytic domain of *IDH2*, including codon 172, were amplified. We applied touchdown PCR, using the standard buffer conditions: it comprised 5 ng of DNA and AmpliTaq Gold DNA Polymerase (Applied Biosystems) run for 16 cycles with denaturation at 95°C for 30 s, annealing at 65 to 57°C (decreasing by 0.5°C per cycle) for 30 s, and extension at 72°C for 60 s in a total volume of 12.5 μl and add 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, ending with at 72°C for 7 min to complete extension.

Direct sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using the statistical software SPSS for Windows, version 17.0 (SPSS Inc, Chicago, Ill). The Mann-Whitney U test, χ^2 test, and Fisher exact test were used to test for association of clinical variables and molecular markers. Survival was estimated by using the Kaplan-Meier method, and survival curves were compared by using the log-rank test. Progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow up. Overall survival (OS) was calculated from the day of first surgery until death or the end of follow up. Univariate and multivariate analyses were performed to test the potential influence of baseline characteristics on survival. The effect of each single molecular marker on PFS and OS was investigated using the Cox proportional hazards model, adjusting for the major clinical prognostic factors, including age at diagnosis (<40 vs ≥40, <60 vs ≥60 years), ECOG performance status score (ECOG PS; ≤1 vs >1), extent of resection (macroscopic [gross] total resection [GTR] vs non-GTR), tumor location (superficial vs deep), MGMT promoter methylation status, chromosome 1p loss of heterozygosity (LOH), 19qLOH, *PTEN* loss, *CDKN2A* loss, *TP53* loss and mutation, *ERBB2* amplification, *EGFR* amplification, *IDH1* and *IDH2* mutation, and adjuvant therapy (with IFN-β vs without IFN-β). Factors with no significant association with survival, at a level of more than 0.05 in the multivariate analysis, were eliminated. The remaining factors in the multivariate proportional hazard model ($P < .05$) were considered to be independent predictors of survival.

To assess for the treatment effects of TMZ with IFN-β versus TMZ without IFN-β for overall survival (OS), the hazard ratio was computed using a proportional hazard model by baseline characteristics in stratified analysis.

RESULTS

Clinical parameters

Between May 2006 and June 2010, 68 consecutive patients newly diagnosed with primary GBM were registered in this study. Their clinical characteristics are summarized in Table 1. This study group comprised 41 men and 27 women aged 12-84 years (median, 55). The median preoperative ECOG PS score at diagnosis was 1 (range, 0-3); the preoperative ECOG PS score was <1 in 45 patients (66.2%). All tumors were located in the supratentorial region: 50 tumors were located in the superficial area (cortical or subcortical area), and 18 were located in deep anatomical structures such as the basal ganglia and corpus callosum. No tumor was noted in the optic nerve, olfactory nerve, and pituitary gland on pretreatment MRI. No tumor dissemination was detected by MRI. Surgical GTR was achieved in 24 patients (35.3%), and 44 patients underwent non-GTR (64.7%). None of the patients had concurrent active malignancy, and the baseline organ function before chemotherapy was as follows: absolute WBC ≥3000/mm^3 or neutrophil count ≥1,500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥8.0 g/dl, AST less than 2.5 × the upper limit of normal (ULN), total bilirubin 2 × ULN, and creatinine 2 × ULN, and electrocardiogram showing no serious

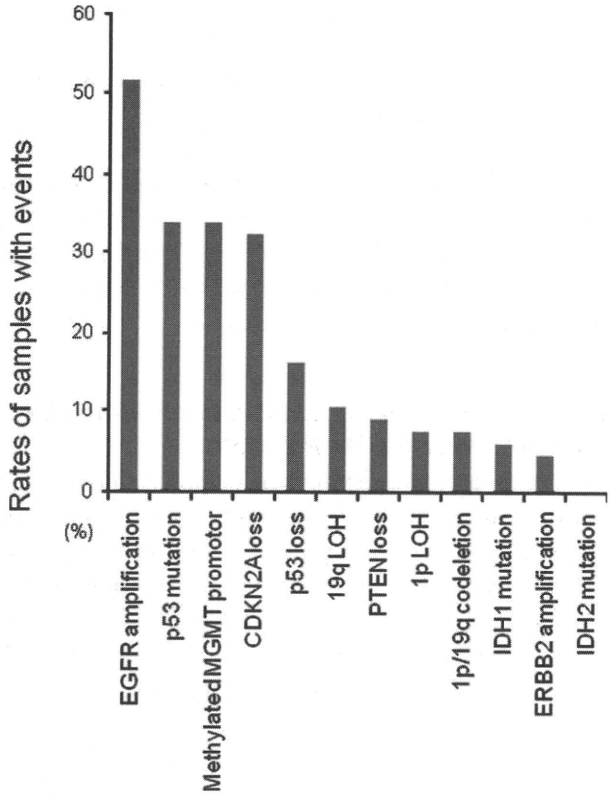

Figure 1. Frequency and pattern of genetic and epigenetic alterations in newly diagnosed primary glioblastoma multiforme (GBM).

arrhythmia and no serious ischemic heart disease. All patients received the standard Stupp regimen,[1] and among these, 39 patients were received combination treatment with IFN-β, as described in the method section.

Frequency of Genetic and Epigenetic Alterations

Of 68 cases, we could obtain sufficient genetic and epigenetic information in all cases. We used direct sequencing for *TP53* and *IDH1/2*. We employed MLPA for the analysis of 1p/19q LOH, loss of *TP53, PTEN* and *CDKN2A*, and amplification of *ERBB2* and *EGFR*. MLPA is a multiplex PCR method that detects abnormal copy numbers of up to 50 different genomic DNA sequences simultaneously. When comparing MLPA to FISH, MLPA not only has the advantage of being a multiplex technique but also one in which very small (50-70 nt) sequences are targeted, enabling MLPA to identify the frequent, single gene aberrations that are very small to be detected by FISH. Furthermore, for the detection of *EGFR* amplification, MLPA can examine exons 1-8, 13, 16, and 22, while previously reported real-time PCR covers only exons 2, 17, and 25. In our preliminary experiments, MLPA was found to be approximately 80% consistent with the real-time PCR method (data not shown). Notably, the methylation status of the MGMT promoter was analyzed by quantitative pyrosequencing technology. Although methylation-specific PCR analysis of MGMT promoter methylation is a widely applicable biomarker for the clinical setting, it is non quantitative and bears a risk of false-positive or false-negative results, especially when the DNA quality and/or quantity is low. Recent attempts to remedy some of these deficiencies have led to the development of an alternative sequence-based approach for methylation analysis, known as pyrosequencing. Pyrosequencing yields continuous methylation values ranging from 0-100%. Based on our comparisons with standard methylation-specific PCR and immunohistochemical study using the anti-MGMT antibody, we determined 14% as the threshold distinguishing unmethylation and methylation of the MGMT promoter in a given tumor.

As indicated in Figure 1 and Table 3, the alterations frequently observed were *EGFR* amplification (51.5%), *TP53* mutation (33.8%), *CDKN2A* loss (32.4%), *TP53* loss (16.2%), methylation of the MGMT promoter (33.8%), and *IDH1* mutation (5.9%). These findings were consistent with those in previous reports.[3,9,20,21]

Clinical, Genetic, and Epigenetic Parameters Associated With Survival in GBM Patients

The median follow-up time was 16.7 months (range, 3.4-46.7 months). The median PFS for all patients was 9.2 months (95% confidence interval [CI], 5.7-12.7). The median OS of all patients was 17.1 months (95% CI, 15.5-18.7) (Figure 2A). The log-rank tests demonstrated that tumor localization ($P = .032$), the MGMT methylation status ($P = .029$), and *TP53* mutation or loss ($P = .035$) were associated with the OS of patients with GBM (Figure 2B-D). These findings were similar to univariate analysis, where deep location ($P = .035$), unmethylated MGMT promoter ($P = .033$) and *TP53* mutation or loss ($P = .038$) were identified as candidate variables for poorer OS (Figure 2). In contrast, well-established prognostic factors such as age, ECOG PS, and the extent of tumor resection did not influence the outcome in this clinical setting. Next, we established multivariate survival models for OS. The model was designed to consider each of these factors without considering the interaction terms. The independent prognostic factors for OS were methylated MGMT promoter ($P = .016$).