

Review Article

MicroRNAs as biomarkers for human glioblastoma: progress and potential

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Abstract

Glioblastoma multiforme (GBM) is the most common malignant glioma. Despite innovative research efforts in tumor therapy, the outcome for most diagnosed patients remains poor; therefore, early diagnosis of GBM is the most effective method for achieving better patient outcomes. In recent years, combined research efforts including cellular, molecular, genetic, and bioinformatics methods have been used to investigate GBM, and the results show that variations in miRNA expression occur in GBM tissues and biological fluids. Some highly stable miRNAs circulate in the blood and cerebrospinal fluid (CSF) of both healthy individuals and diagnosed patients, thus raising the possibility that miRNAs may serve as novel diagnostic markers. In addition, increased understanding of the miRNA and mRNA interactions involved in GBM progression may lead to discovering predictive biomarkers, some of which are clinically relevant for targeted therapy and predicting prognosis. However, as this field is relatively new, some studies have yielded conflicting results. To progress in the field, different advanced techniques must be combined, including bioinformatics methods and molecular and cellular techniques. In addition, we must overcome the various challenges in non-invasive GBM biomarker detection. Here, we discuss the progress and potential of miRNAs as biomarkers for GBM and related signaling pathways. Studying the clinical relevance and applicability of these biomarkers may alter GBM patient diagnosis and treatment.

Keywords: glioblastoma; miRNA; biomarker; diagnosis; bioinformatics; non-invasive detection

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Introduction

Although glioblastoma multiforme (GBM) is rare, it remains the most common primary malignant brain tumor. The incidence ranges from 4.67 to 5.73 cases per every 100000. Even after patients have undergone chemo and radiation therapy, the median survival time is 14.6 months. The five-year survival rate is only 0.05% to 4.7%, making GBM the most common, as well as the most lethal primary brain tumor^[1]. Based on histological similarities in cell origins and their differentiation, GBM was previously classified as either GBM isocitrate dehydrogenase (IDH)-wildtype, accounting for about 90% of cases, or GBM IDH-mutant type, accounting for the remaining 10% of cases^[2,3]. Temozolomide (TMZ), a standard chemotherapy agent that targets GBM, acts by methylating and crosslinking DNA. O6-methylguanine-DNA methyltransferase (MGMT) is an enzyme that reverses this crosslinking. Promoter methylation and MGMT gene silencing is expected to respond better to TMZ, resulting a higher survival rate.

Therefore, the MGMT expression level has become important for predicting disease prognosis^[4,5]. Using TMZ to treat GBM has been disputed. In one clinical trial, patients with GBM with MGMT methylation who were treated with TMZ demonstrated improved survival compared to patients with GBM without MGMT; however, in another trial, the survival rate was lower^[6]. In addition, other oncogenic alterations, such as platelet-derived growth factor (PDGF), epidermal growth factor receptor variant III (EGFRvIII), neurofibromatosis type 1 (NF1), and fibroblast growth factor receptor (FGFR), are being investigated as biomarkers for predicting prognosis and as targets for molecular therapies^[6]. Therefore, the roles of traditional biomarkers such as MGMT as well as emerging biomarkers remain uncertain, and the sustained low survival rate for GBM patients demands for new prognostic indicators to aid in clinical decision-making. Developing more effective diagnostic and therapeutic strategies based on a biologically and clinically relevant disease sub-classification system has become critical. The importance of biomarkers in identifying tumor-specific treatment and disease monitoring has been demonstrated, as it is often infeasible to biopsy the tumor tissues^[7].

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MicroRNAs (miRNAs) are small RNAs, approximately 22 nucleotides long, which do not encode proteins, but bind to messenger RNAs (mRNAs) and play important roles in gene regulation^[8-10]. Previous studies have shown that miRNA expression in tumor tissues differs from that of normal control tissues. Profiling these miRNA expression differences can help to further classify GBM^[11-13]. Furthermore, several studies have shown that a combination of miRNA expression profiles and the use of bioinformatics methods (such as clustering) may produce more accurate histological and prognostic tumor classifications compared to results based solely on mRNA expression. Extensive research has been focused on multiple miRNAs that dysregulate expression in GBM. In previous studies, patients with differing miRNA expressions had different prognoses and responded to different treatments. Therefore, miRNAs may help classify, diagnose and predict the clinical course for patients with brain tumors^[12-15].

In this review, we focus on the clinical characteristics of GBM subclasses and the validity of circulating miRNA as potential prognostic biomarkers for GBM based studies on human GBM tumor specimen. These studies aim to characterize and stratify a patient's GBM based on individual genetic profiles to allow more personalized treatment.

The developmental classification of glioblastoma by miRNAs

Glioblastoma is a malignant heterogeneous brain tumor; thus, the patient's clinical manifestations and treatment responses differ based on the differences in genetic signal networks. Therefore, accurately classifying GBM helps in therapy and prognosis. Due to the unfavorable prognosis of glioblastoma, it is critical to investigate more effective diagnostic and therapeutic strategies based on the biological and clinical disease classification system. Recent studies have proposed several mRNA-based classification systems for GBM. One widely reported gliomas classification system includes proneural, neural, classical, and mesenchymal subtypes^[16]; however, previous studies have shown that expression-based classifying using miRNA profiles may more accurately classify histological and prognostic samples than those based on mRNA profiles^[11].

In this section, we review the advances made in identifying specific miRNA expression profiles in GBM subgroups. An analysis by Henriksen *et al* showed that differentially expressed 161 miRNA pattern clustering defined two groups of GBM patients based on clinical data, which corresponded to either long-term (~1500 days) or extremely short-term (~550 days) survival. These two miRNA groups had significantly different expression levels, with a two-fold change as the cut off (long-term cluster *vs* extremely short-term cluster)^[13]. In Table 1, columns one and two show the portions of these 161 miRNAs that were confirmed by other studies, but the extremely short-term survival data are not shown, as no comparison data were obtained from other studies. Moreover, Kim *et al* analyzed 261 miRNA expression profiles in gliomas from the Cancer Genome Atlas (TCGA) and identified five

clinically and genetically distinct glioblastoma subclasses, each relating to a different neural precursor cell type. These subclasses include radial glia, oligoneuronal precursors, neural precursors, astrocyte precursors, and neuralmesenchymal precursors^[11]. In comparing the four subtypes based on mRNA expression alone, we found that oligoneuronal precursors corresponded to proneural subtype due to IDH1 mutations, neuralmesenchymal precursors corresponded to mesenchymal subtype due to NF1 mutations, and radial glia may correspond to classical subtype due to high levels of FGFR expression. The proneural and mesenchymal subtypes have consistently been confirmed in other gene expression profiling studies^[17], while other GBM subtypes or precursors have not. However, when considering the cell subtypes of each tumor and their mixed cellular states, the GBM classification is further complicated by the glioblastoma stem cell (GSC) subpopulations maintaining their transcriptome heterogeneity. Based on differences in the patients' race, age, treatment response, and survival rates, each determined subclass was genetically distinct. Kim *et al*'s study on 121 selected miRNAs showed highly varied expression that was closely related to patient survival or previously associated with neural development. In Table 1, columns 4 and 5 show that these 121 miRNAs identified five miRNA clusters and five distinct glioblastoma subclasses. Based on patient survival rates, these five GBM subclasses can be divided into two subclasses: the long (>2400 days) survival subclass (oligoneuronal, neuralmesenchymal, and radial precursors), and the short (~1500 days) survival subclass (neural and astrocyte precursors). This was consistent with the data from Henriksen's study, shown in Table 1, as the short survival subclass in Kim's study corresponded to the long term in Henriksen's study. Furthermore, Li *et al* used microarrays and non-negative matrix factorization on the dataset and RNASeqV2 from the TCGA-Assembler, containing 169 GBM and 5 normal samples. Four GBM subgroups were identified from this study. In Table 1, columns 6 and 7 show the differential expression in these four groups. In addition, based on patient survival rates, group 1, 2, 3, and 4 all belong to the short-term survival subtype in Kim's study^[18]. In Table 1, group 2 and 3 were put into astrocytic precursor group, as indicated by miR-770 and miR-24; however, there was relatively less consistency in Li *et al*'s study compared with other studies after considering both miRNA and mRNA expression. The reason for this discrepancy may be that their survival terms differed from those to which we compared their survival terms. All survival terms in Li's study should correspond with the long-term survival in Henriksen's study or the short survival in Kim's study.

Several key miRNAs mentioned above from the short-term survival cluster were confirmed by other studies. Srinivasan *et al* studied the ten miRNA signatures to predict GBM patient survival, in which seven risky miRNAs were discovered, including miR-31↓, miR-146b↑, miR-148a↑, miR-193a↑, miR-200b↑, miR-221↓, miR-222↓, were discovered. As shown by arrows, four were over-expressed and three were under-expressed. These miRNAs were defined as risky because of

Table 1. The different classification of GBM.

Survival days	miRNAs ^{[13]*}	miRNAs ^[19]	Groups	miRNAs ^[11]	Groups	miRNAs ^[18]
~1500 Short survival subclass	miR-204, 222-3p, 24, 200b-3p, 29b-3p, 29c-3p, 34a-5p, 146b-5p, 29b-1-5p, 29b-2-5p, 770-5p	miR-31, 222, 148a, 221, 146b, 200b, 193a	Astrocytic precursors	miR-191, 425-5p, 192, 200b, 200a, 29c, 29b, 29a, 27a, 24, 23a, 223, 34a, 142-3p, 146b, 22, 21, 221, 222, 155, 148a, 193a, 204, 31, 34b, 210, 193b, 339	Group2	miR-770-5p, miR-1184, miR-133a, miR-516a-3p, miR-133b
	miR-23b-3p, 487b, 128-3p, 137, 504, 377-5p,			miR-565, 487b, 504, 128a, 128b, 136, 137, 376a, 338, 218, 219, 377, 376a*, 127, 124a, 7, 23b, 27b, 582	Group3	miR-220a, miR-492, miR-626, miR-24-1a, miR-489
~2400 Long survival subclass		miR-20a, 106a, 17-5p	Oligoneuronal precursors	miR-26a, 135b, 196a, 196b, 10b, 9*, 9, 182*, 130a, 20a, 25, 424, 340, 92, 96, 17-3p, 106a, 17-5p, 182, 18a, 183, 19a, 19b, 181a, 181b, 149, 345, 505, 362	Group1	miR-147b, miR-1269, miR-744, miR-483-5p, miR-1207-5p,
				miR-623, 564, 629, 630, 765, 671, 638, 663, 801, 759-3p, 452, 451, 206, 370, 490, 486, 492, 493-3p, 513, 494, 575, 572	Group4	miR-516a-3p, miR-133b, miR-1289, miR-214
				miR-99b, 125a, 92b, 30a-5p, 125b, 100, 99a, let-7c, let-7d, 26b, 98, 195, 135a, let-7e, let-7i, let-7b, let-7a, 335, 126*, 126, 146a, 143, 145		

*Short survival (~1500) vs extremely short survival (~500), miRNAs in other columns are GBM or gliomas vs normal control.

the association between their expression and patients survival^[19]. Those seven miRNAs shown in column 3 of Table 1 were overlapped by astrocytic precursors and the short-term survival cluster defined by Kim group. Three miRNAs were protective miRNAs (miR-17-5p↑, miR-20a↑, miR-106a↑) in tumors from glioblastoma patients with shorter or median survival. If those three miRNAs were in up-regulated condition, they would be found in the long-term cluster, which have been confirmed by Kim group, as shown in columns 3 and 5 of Table 1. Recently, a miRNA cluster including miR-23a, miR-27a, and miR-9-3p (miR-9*) was determined to discriminate mesenchymal and proneural subtypes of primary GBM. Here, mesenchymal and proneural subtypes were defined by Verhaak's study to be in their short-term and long-term survival subgroups, respectively^[16]. Column 5 of Table 1 shows miR-23a and miR-27a in the short-term cluster and miR-9* in the long-term cluster. Furthermore, miR-23a and miR-27a were defined as high-confidence miRNAs in GBM using a bioinformatic pipeline for miRNA and mRNA profiles, combining with cellular and molecular techniques for GBM tissues with short-term survival rates and cell lines^[20]. However, less overlapping was found between the results from Li group and the other groups, possible due to the clustered miRNAs were based on different clinic-pathological mechanisms, including

patient survival terms, neural development, or differentially expressed genes.

miRNA expression and regulation in glioblastoma tissues

In addition to the classification studies noted above, many other studies have analyzed individual miRNAs as potential diagnostic biomarkers by comparing tumors to normal tissues. miRNAs are involved in major GBM regulatory pathways, including proliferation, differentiation, apoptosis, migration, and invasion^[21]; thus, these miRNAs have been associated with patient survival and therapeutic treatment response in previous studies^[15, 18].

Several miRNAs regulate GBM cell proliferation. In previous studies, only miR-21 was consistently up-regulated in GBM tumor tissues (as well as ovarian cancer, lung cancer, and head and neck cancer)^[22-26]. Higher miR-21 expression was associated with low apoptosis, high proliferation, and worse prognosis and patient survival in GBM^[24, 27]. miR-93 was likely associated with cell proliferation and angiogenesis in GBM. Its expression levels were up-regulated in GBM tissues, and higher miR-93 levels were associated with early tumor invasion and poor survival^[28, 29]. Many studies have demonstrated that high miR-196 expression correlates with poor prognosis in GBM patients by inducing cell proliferation

and inhibiting cell apoptosis^[30, 31]. In addition, miR-335 has been found to induce tumor proliferation, differentiation, and invasion by interacting with the cAMP/protein kinase A pathway. Therefore, increased miR-335 expression was often seen in GBM tissues, and miR-335 overexpression was associated with poorer prognosis in GBM^[32-34].

miR-7 and miR-34a were found under-expressed in GBM tissues, which correlated with a poorer patient prognosis. Since miR-7 acts as a tumor suppressor by inhibiting epidermal growth factor, decreased miR-7 expression levels were found in GBM tissues, and lower miR-7 levels were associated with more GBM invasive subtypes and poorer outcomes post-treatment^[35, 36]. Similarly, miR-34a, which potentially interacted with the p53 pathway and as a tumor suppressor, had decreased expression in GBM tissues. In several studies, patients with lower miR-34a expression showed poorer survival rates^[37, 38]. In addition, miR-27a had decreased expression levels in GMB tissues, but its targets are involved in cell cycle progression, and its effect is mainly oncogenic. Paradoxically, higher miR-27a expression levels were found in lower grade GBM, and patients had better prognosis with higher miR-27a expression. It has been postulated that cell cycle dysregulation is driven by miR-27a, so its over-expression was less aggressive than other dysregulations^[39]. By contrast, miR-19 had increased expression levels in GMB tissues and is postulated to play a role in oncogenesis; thus, miR-19 overexpression was found in higher grade GBM, resulting in a poorer prognosis^[14, 40]. Interestingly, miR-125b is controversial in tumor regulation, as it plays oncogenic and tumor suppressing roles in different tumors. miR-125b blocks apoptosis by interacting with the p53 pathway and suppressing cell proliferation by interacting with proteins that regulate cell proliferation such as Bcl-2 in GBM^[41, 42]. Therefore, studies have demonstrated decreased miR-125b expression levels^[43, 44]; while others have shown increased miR-125b expression levels with poorer associated prognosis, although the cellular mechanism is unclear^[35, 45].

miRNAs respond differently to TMZ, which is a first-line treatment for GBM. miR-181 expression levels play a role in disease progression and standard treatment response. Decreased miR-181 expression was observed in patients who responded well to TMZ^[46], while higher miR-181 expression was associated with faster disease progression among GBM patients^[47]. Similarly, miR-195 was postulated to suppress tumors by interacting with cyclin-D and promoting cell entry into S-phase. As hypothesized, miR-195 expression was decreased in GMB tissues^[48]. However, miR-195 was shown to over-express in patients with TMZ-resistant GBM, which indicates its potential role in directing tumor specific treatment^[47, 49]. Although the molecular mechanism of resistance is unclear, miR-455-5p over-expression was also associated with TMZ-resistant GBM^[50]. In addition, miR-210 was also dysregulated in GBM. When miR-210 was overexpressed, it promoted oncogenesis, resisted to TMZ, and led to lower patient survival rate^[14, 30, 31].

Some miRNAs are involved in several regulatory pathways. For example, studies have shown that miR-218 interacts with

multiple pathways to inhibit cellular proliferation, tumor invasion, and tumor migration. These studies demonstrated that miR-218 levels were decreased in patients with more invasive and aggressive GBM types^[29, 51, 52].

miRNA expression in GBM patient peripheral fluids

miRNA profiles in the tumor tissues may help determine tumor grades, disease prognosis, and therapeutic targets, but characterizing miRNA profiles in a patient's peripheral body fluid is more feasible for non-invasively diagnosing GBM. miRNA profiling in peripheral fluids is much less invasive than repeatedly taking tumor tissues biopsies. The relationship between miRNA existing in the peripheral fluids and miRNA expression in the GBM tissues is unclear; thus, we listed several miRNA expressions for each condition shown in Table 2. In Table 2, arrows indicate the up-regulation or down-regulation of GBM miRNAs with consistent results. Several studies have shown that miR-128 levels were increased in patient peripheral blood, although it was decreased in GBM tissues. Thus, the level of miR-128 in blood may serve as a biomarker for detecting GBM. However, it was postulated that miR-128 levels can be falsely elevated due to the miRNA that is released from red blood cells^[32, 33]. miR-21 level was also increased in patient serum, brain tissues, and exosomes or EV from peripheral fluids, as shown in Table 2. However, the upregulated miR-21 has been reported in several cancers, including breast, hepatocellular, colorectal, gastric, ovarian, and cervical cancers^[25]; therefore, it may be impossible to detect GBM by measuring serum miR-21 or miR-128 levels. In addition, miR-342-3p levels have shown to be down-regulated in GBM patients' peripheral plasma, although it is unclear if they were associated with any cellular mechanisms^[32, 34]. Studies have also shown decreased miR-125b and miR-497 expression levels in GBM patient serum compared to normal patient serum, although conflicting results were found regarding miR-125b expression in GBM tissues (Table 2). miR-125b and miR-497 may be associated with suppressing tumors and regulating cell proliferation^[33, 43, 53], and therefore may be potential candidates for detecting GBM.

As mentioned above, it is difficult to predict GBM from only one miRNA. To solve this, several miRNA panels in the peripheral fluids may diagnose the disease well. A miRNA panel demonstrated that miR-576-5p, miR-340, and miR-626 overexpression and miR-320, let-7g-5p, and miR-7-5p underexpression in GBM patient sera tested by the microarray method^[54]. In a recent study, researchers established two miRNA panels to predict the feasibility of an estimated two-year survival period among GBM patients. The first panel was comprised of three serum miRNAs: miR-106a-5p, miR-182, and miR-145-5p, in which up-regulated miR-106a-5p and down-regulated miR-182 and miR-145-5p were significantly associated with short-term (two years) survival of GBM patients. The second panel was comprised of five serum miRNAs: miR-222-3p, miR-182, miR-20a-5p, miR-106a-5p, and miR-145-5p^[55]. In this panel, up-regulated miR-222-3p, miR-20a-5p, and miR-106a-5p, and down-regulated miR-182 and miR-145-5p were

Table 2. The potential biomarkers of GBM.

miRNA	Tissues	Exosome or EV		Peripheral fluids	
		From blood	From CSF or CM*	Plasma or Serum	CSF
miR-21	↑[26]		↑[58]	↑[34, 57, 72]	↑[69]
miR-221	↑[26]		[68]	↑[54]	
miR-15a/b	↑[20, 43]			↑[32]	↑[69]
miR-182	[7]			↓[55]	
miR-128-a/b	↓[29]			↑[32]	↓[69]
miR-20a	↑[9][13]	[66]	[58]	↑[55]	
miR-125b	[20]		[58]	↓[43]	
miR-106-a/b	[11, 19, 20]		[58]	[44][55]	↑[69]
miR-17	[13, 7, 19]	[56][58]	[58]	↑[54]	↑[69]
miR-27a	↑[20][7]	[56,58]			↑[69]
miR-99-a/b	↑[11, 13, 20]		[58]	↑[54]	
miR-130-a/b	[11, 20]		[58]	↑[54]	↑[69]
miR-25	[11][15]		[58]	↑[54]	↑[69]
miR-23a	↑[11, 20]			↑[54]	↑[69]
miR-10b	↑[11, 20]			↑[57]	↑[69]

*Condition medium, ↑Up-regulation, ↓Down-regulation.

correlated with a lower probability of two-year disease-free survival^[55]. Because miR-20a and miR-106a were consistently expressed in GBM tissues, they become important potential biomarker candidates. Other studies supported down-regulated miR-106a as a potential biomarker for short-term GBM survival^[19]. Recently, down-regulated miR-106a was found by a bioinformatic pipeline using transcript expression profiles from GBM tissue, and the result was validated by real-time PCR in GBM tissues and cell lines when the prostate cancer 3 (PC3) cell line was used as the control^[20]. Three miRNAs that were over-expressed (miR-17-5p, miR-20a, miR-106a) in tumors from glioblastoma patients with shorter or median survival, were discovered^[19] and confirmed by Kim's study; however, there were conflicting reports. Some stated that miR-106a-5p and miR-20a-5p overexpression were associated with tumor invasion and poorer prognosis, suggesting that these two miRNAs were oncogenes. Others stated that miR-106a, miR-182*, miR-20a-5p, and miR145 were selected as short-term survival signatures of miRNA in GBM tissues. These conflicting results may be due to different definitions for the survival terms. In addition, miR-145-5p in the second panel suppresses tumor; thus, low expression of miR-145-5p was associated with lower survival rates. By contrast, miR-222-3p promoted tumor growth, so its overexpression was also associated with poorer outcomes and lower survival rates^[55]. The role of miR-182 in GMB development is controversial. One study found that down-regulated miR-182 was associated with poorer prognosis, but another study that included patients with all subtypes of glioma found that miR-182 overexpression was a potential poor prognostic marker^[55, 56].

Elevated levels of some miRNAs probably reflect the anti-tumor effect of GBM therapy. High levels of miR-10b and miR-21 were observed in most GBM patient serum during the bevacizumab (BVZ) treatment period compared to the pre-

treatment levels. Furthermore, the miR-10b and miR-21 levels expressed were negatively correlated with increased tumor diameters in the BVZ-treated group but not in the TMZ-treated group^[57]. Therefore, monitoring these miRNAs in the circulation could aid in predicting their therapeutic effects.

miRNA expression in GBM patient exosomes

Exosomes are a special type of extracellular vesicle with a spherical shape and lipid bilayer of approximately 30–200 nm. They are sometimes referred to as extracellular vesicles (EVs) or micro-vesicles (MVs) depending on their size. The term, exosome, typically refers to EVs of 50–200 nm, while the terms, EV or MV, refer to EVs > 200 nm^[58]. They are believed to have originated from multi-vesicular bodies during membrane recycling. Exosomes contain proteins, phospholipids, nucleic acids, and other materials that can be released into the extracellular environment to maintain homeostasis, regulate the immune system, and participate in intercellular exchange of proteins and genetic material^[59–62]. Studies have shown that exosomes released by GBM tissues have similar components to the tumor tissues themselves, and the contents released by exosomes are likely to influence the host's immune response, such as resisting chemotherapeutic agents, promoting tumor proliferation, and facilitating tumor invasion. Therefore, the exosome's miRNA expression profile is a potential biomarker for providing information on tumor grades and prognosis, which would then allow the design of better tumor specific therapies^[58, 63–67].

Recently, high levels of miR-451 and miR-21 were found in GBM-EVs that were isolated from cultures of human glioblastoma cells. Many RNA species are produced within tumor cells specifically for export out of the cells. miR-451 is critical in glioma cell proliferation and migration. Selective export of miRNAs can indicate whether a certain miRNA is tumor-

specific or not. In this case, selective export of miR-451 was not found to be tumor-specific, as it was also enriched in MV from non-neoplastic cell lines. miR-451 has been shown to inhibit the adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, which is normally regulated for metabolic needs. Thus, miR-451 levels in exosomes released by GBM are fine-tuned to serve the tumor. Previous studies have demonstrated the process of the exosomes containing miR-451 and miR-21 being absorbed by glial cells adjacent to GMB tumor cells^[67, 68]. miR-21 overexpression in patient serum has been reported consistently, and its exosome level is increased 10-fold compared to the control group^[66, 68]. miR-21 upregulation in GBM was consistent in brain tumor tissues, blood, CSF, and exosomes, as shown in Table 2. However, miR-21 is sensitive to most cancers. As a general rule, for a biomarker to be effective, the biomarker must be specific for that cancer. Therefore, it is difficult to use only miR-21 as a biomarker for detecting GBM. miR-10b is expressed in only tumors not in normal brain tissues, while, miR-21 is expressed in both. Thus, when analyzing different CSF specimens, a positive correlation was observed for miR-10b, as it was expressed more in brain tumors^[69], suggesting that miR-10b may indicate advanced primary and metastatic brain cancers.

In addition, miR-320 and miR-574-3 were both elevated significantly in exosomes from GBM patient sera and could serve as potential diagnostic biomarkers^[70]. Although miR-320 has important roles in different cancer types, such as breast and colon cancers, the combination of miR-320 and miR-574-3p was found consistently in exosomes from GBM patient sera. It was postulated that the combination of these two miRNAs could serve as a biomarker for diagnosing GBM. In the study conducted by Zhang *et al*, high levels of miR-221/222 were associated with shorter survival, possible because miR-221/222 overexpression downregulates protein tyrosine phosphatase μ (PTP μ) expression. PTP μ is strongly associated with preventing GBM growth by lowering GBM cell migration^[64]. Other miRNAs that are down-regulated in multiple types of cancer, including miR-15, miR-16, and miR-146b, are also down-regulated in GBM exosomes^[64, 65, 69, 70]. Again, while one miRNA alone may not be an adequate biomarker, it may be possible to use a combination of miRNAs that are unique to GBM to serve as a biomarker. In short, the continuing search for biomarkers unique to GBM has merely begun. One limitation of many of the studies discussed above is in differentiating between the different subtypes of GBM. Using miRNAs to do so is a possible path for future exploration and study.

The challenges for miRNAs as biomarkers in GBM

Given the previously discussed roles of miRNAs in GBM classification and diagnosis, miRNAs have attracted a great deal of attention as potential biomarkers that may facilitate patient management decisions. Thus far, the possible advantages of miRNAs as biomarkers include non-invasive diagnosis, easy detection, and effective classification. Due to the presence of miRNAs in bio-fluids, patients can be diagnosed non-invasively. miRNA characteristics make them much more effec-

tive as biomarkers compared to traditional biopsies. Detecting miRNAs in bio-fluids is relatively easier than detecting protein or metabolites in brain tissues due to significantly improved tools such as RT-qPCR, next-generation RNA sequencing, and micro-array platforms. In addition, due to miRNA stability at room temperature and with simple handling, miRNAs may be more suitable for biomarker detection compared to other protein or metabolic blood biomarkers. Furthermore, based on preclinical studies, miRNA signatures could be unique to the pathways that are altered in the diseased states, which is the fundamental basis for a biomarker. Unfortunately, extensive numbers of conflicting reports exist that speculate on the specificity of miRNAs for specific diseases. Many reasons can cause a lack of reproducibility among studies, such as different bio-fluids, different internal controls, and different detection methods. Here, we divide these into two categories, technique challenges and basic challenges, and discussed each separately.

The technique challenges defined in this paper can be resolved by improving or changing the use of different detection methods. Some technique challenges, including the types of bio-fluids, methods of collecting the bio-fluid and purifying the RNA, and tools for detecting miRNAs, affect the detected miRNA results. These technique challenges make it difficult to use miRNAs as biomarkers in GBM. Several articles have discussed that researchers should pay more attention to choosing the correct bio-fluids, using suitable collection and storage methods, and purifying RNA carefully^[25, 71]. Thus, we will not discuss these again. Rather, we will discuss the primary methods for detecting miRNAs: RT-qPCR, microarray, and next-generation sequencing (NGS). Microarrays can assess genome-wide profiling of many miRNAs in blood and brain tissues to identify candidate biomarkers for diagnostic and prognostic purposes in GBM patients, but they cannot be used for absolute quantification as they are less sensitive and specific than RT-qPCR. In the NGS approach, knowledge of target miRNAs and specific probes or primers is unnecessary, thus enabling investigators to assess unknown miRNAs. However, NGS analysis requires specific miRNA bioinformatics support, and its required time and cost are longer and higher compared to RT-qPCR. After exploring these three methods, we recommend using RT-qPCR as the primary method for detecting miRNAs in diagnosing any cancer including GBM.

These defined basic challenges can be overcome by further exploring the regulatory mechanisms of miRNAs in GBM development and progression, such as similar miRNAs in various diseases, many miRNAs in one disease, and several miRNAs in different statuses of one disease. Resolving these challenges requires more research. New techniques that may help to explore and overcome these challenges include microarray profiling, high-throughput sequencing, and bioinformatics pipelines. As mentioned above, the increased miR-21 and miR-10b expression was detected in both GBM tissue and sera. In addition, the combination of these two miRNAs in the serum allowed MRI detection to reveal the patients' response to BVZ. Most patients with high serum expression of these

two miRNAs showed significantly reduced tumor diameters during BVZ treatment. By contrast, in the TMZ treatment group, no significant correlations between changes in these two miRNA levels and tumor dimension or clinical response was observed^[57]. Thus, this fundamental research revealed that the combination of miR-10b and miR-21 could serve as a biomarker for GBM clinical treatment using BVZ. In addition, miR-21 is reported to be highly expressed in most cancers^[25]; however, it lacks specificity as a biomarker for GBM, but can be used in a biomarker panel when combined with miR-10b.

Bio-fluid miRNAs have high potential as GBM biomarkers; however, further investigations are needed to answer the lack of reproducibility and specificity, and to determine possible contamination sources such as miRNAs from blood cells. In addition, coordinating technology and achieving consistent data can transform miRNAs into reliable biomarkers for use in clinical settings.

Conclusion

GBM is one of the most aggressive primary brain cancers with a median survival of 15 months. Identifying suitable biomarkers would greatly aid clinicians in diagnosing and creating specific therapies. Multiple miRNAs have been found to be dysregulated in GBM tissues and play key roles in oncogenesis, progression, and invasion. From this review, two things were clear: 1) Several miRNA panels may classify GBM more accurately than before. As mentioned above, several recent studies suggest that the panel of ten miRNA signatures in GBM tissues successfully indicated the GBM subtype that was correlated to short-term survival, which was also defined as the astrocyte precursor subtype. Additionally, the panel of three miRNAs distinguished the proneural subtype from the mesenchymal subtype, which was back up by several studies. 2) Efforts are needed to find effective GBM biomarkers. To do so, we must overcome the technical and basic challenges in using miRNAs as GBM biomarkers. Multiple studies have shown miRNAs are great candidates as diagnostic and prognostic biomarkers. However, repeatedly performing biopsies on tumor tissues is often infeasible. Therefore, identifying miRNA profiles in peripheral fluids and exosomes is a key step in classifying tumor subtypes, designing tumor specific therapy, and eventually improving patient survival.

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