A Signature of Three MicroRNAs is a Potential Diagnostic Biomarker for Glioblastoma

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Research Article

Keywords: Biomarker, Brain tissue, Glioblastoma, Diagnosis, miRNA

Posted Date: November 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1048138/v1>

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Abstract

Background: Glioblastoma (GBM) is by far the most common primary malignant neoplasm of the central nervous system. In spite of some progress in diagnosis and treatment, GBM patients still have a poor prognosis. Hence, to improve GBM diagnosis, finding novel non-invasive biomarkers, e.g., miRNA-based biomarkers, is of importance.

Methods: A pair of 50 GBM and healthy samples were used. The expression of each candidate miRNA (i.e., hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p) was measured using quantitative reverse transcription PCR. To show the roles of each miRNA and their biological effects on GBM development, *in silico* tools were used. Receiver operating characteristic (ROC) curves were performed to assess the diagnostic accuracy of each miRNA; the possible association of their expression with clinicopathological characteristics was also analyzed. To find out any association between the miRNA expression and GBM prognosis, *in silico* tools were used.

Results: We showed the downregulation of hsa-let-7c-5p and hsa-miR-206-5p, and upregulation of hsa-miR-1909-5p in GBM tumor compared to healthy samples. No association was detected between the expression of each candidate miRNA and 'sex'. Except for hsa-let-7c-5p, other miRNAs did not show any correlation with 'age' status. ROC curve analysis showed that the signature of candidate miRNAs is a potential biomarker distinguishing between GBM and healthy samples. Only hsa-miR-206-5p showed the association with poor prognosis in GBM patients.

Conclusions: We demonstrated that the dysregulation of three candidate miRNAs may be used as a biomarker for GBM diagnosis. These results are beneficial in clinical management of GBM patients.

Introduction

As the most frequent primary brain tumor as well as the most aggressive subtype of glioma, glioblastoma (GBM) is characterized with a low rate of survival (less than 12 months)^{1,2}. Magnetic resonance imaging (MRI) is usually used for initial diagnosis for GBM, however, further prognostic information is gathered using histopathological and molecular analysis, most of which are invasive³. Surgical resection followed by radiotherapy is still the safest and standard-of-care treatment for GBM⁴. In low-grade glioma with negligible tumor size, MRI cannot make an accurate diagnosis⁵. Furthermore, confounding factors—e.g., pseudo-progression or pseudo-response—can complicate the determination of tumor status⁶. On top of that, as doing biopsies several times requires invasive intracranial neurosurgery⁷, they are not feasible in GBM. Also, due to the intratumoral heterogeneity, GBM biopsies are prone to sampling error⁸.

To address these limitations, using biomarkers is recommended. Biomarkers can *ipso facto* help us to better diagnose and monitor GBM patients at different stages of tumorigenesis⁹. Among various biomarkers, non-coding RNAs—especially miRNAs—have attracted some attention^{10–14}. MicroRNAs

(miRNAs or miRs) are a small group (18–25 nt) of non-coding RNA that functions as a gene expression regulator at the post-translational level^{15,16}. In other words, miRNAs function by binding to their target mRNAs and suppressing the protein expression^{10,16}. These molecules are the potential biomarkers for different cancers^{12,13}. They play critical roles in cancer biology through controlling signaling pathways that make a contribution to tumor growth, angiogenesis, and immune invasion^{10,17}. Aberrant expression or dysregulation of miRNAs set stages for malignancy in glioma cells and may take a center stage in GBM development¹⁸. Furthermore, they are versatile (e.g., they are detectable in blood and plasma) which makes them an appropriate biomarker for tumor diagnosis, therefore, they may represent the cellular status of the derived tissues that can be exploited for diagnostic purposes¹⁷.

Herein, we aimed to examine whether a signature of three candidate miRNAs (i.e., hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p) can be a diagnostic biomarker distinguishing GBM from healthy tissues or not. We selected the candidate miRNAs based on literature reviews and data mining for those of miRNAs that maybe actively involved in GBM development or pathogenesis. Furthermore, we conducted an investigation to show the possible relationships between each miRNA expression pattern and the patients' clinicopathological features. To assess biomarker possibility, a receiver operating characteristic (ROC) curve analysis was used. Besides, to show probable targets and the functions of each miRNA, *in silico* analysis was undertaken.

Material And Methods

Patients

Formalin-fixed paraffin-embedded (FFPE) samples were obtained from GBM patients who were admitted by Imam Khomeini Cancer Institute, Tehran, Iran. The Ethics Committee of Islamic Azad University, Damghan, Iran, approved the study protocol, and all donors voluntarily gave their written informed consent before sampling.

Fifty patients with a definite histopathological diagnosis of GBM were included. They were subjected to surgery to remove injured tissues. As a common clinical procedure, MRI data were generated for each patient. Volumetric tumor analysis was carried out by an experienced neuroradiologist who was blinded to the clinical or miRNA outcomes. The samples were assessed histopathologically by experienced neuropathologists based on different criteria based on the World Health Organization for the histologic grade¹⁹.

GBM tissues were collected using standard operating procedures and in total, a pair of 50 tumor and healthy samples were collected from the patients and healthy individuals, respectively. All patients were newly diagnosed GBM, while healthy controls (n = 50) opted from whom with no positive history of brain pathology or malignancy; these people were subjected to surgery for different reasons in addition to malignancy, e.g., mild traumatic head injury.

RNA isolation and quality assessments

FFPE blocks were cut and the total RNA was isolated using TRIzol reagents (Invitrogen, VIC, Australia). Noticeably, to solubilize and remove the paraffin, the samples were washed several times using xylene. The RNA concentration was measured using the NanoDrop 2000c (Thermo Fisher Scientific, CA, USA) and its integrity was verified using 2% gel electrophoresis. To cut down on any probable DNA contaminations, the samples were treated with RNase-free DNase (Ambion, Austin, TX, USA).

Candidate miRNA and experimental validation

We selected the candidate miRNAs based on the literature reviews and data mining for those miRNAs that may contribute to GBM development. Several criteria were considered embracing (i) the candidate miRNAs should have been identified in a dysregulated and detectable manner in human GBM, (ii) each candidate miRNA must have been annotated straightforwardly in miRBase version 22.1, and (iii) they might have been contributed to different signaling pathways in GBM development or pathogenesis. By accepting these criteria, hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p were nominated.

Complementary DNA (cDNA) synthesis and RT-qPCR

Poly-(A)-tailing and cDNA synthesis were performed using MiR-Amp Kit (ParsGenome, Tehran, Iran). The expression of each candidate miRNA was evaluated with RT-qPCR that was carried out on an ABI StepOne Sequence Detection System (Applied Biosystems, NSW, Australia) according to the conditions that have been reported in previous studies^{11,20-22}. To assess the specificity of PCR, melt curve analysis was used.

To normalize the relative expression of each candidate miRNA, U48 snRNA was utilized. To calculate the fold changes, we used the $2^{-\Delta\Delta Ct}$ method²³. The primer sequences were as follows: *U48*-forward: 5'-TGACCCAGGTAAGTCTGAGTGTGT-3', and *U48*-reverse: 5'-GCGTCGACTAGTACAAGTCAAG-3'; miR-let-7c-5p, forward: 5'-GGTGAGGTAGGTTGT-3', and reverse: 5'-GTGCAGGGTCCGAGGT-3'; hsa-miR-206-5p: forward: 5'-GAATGTAAGGAAGTGTGTG-3' and reverse: 5'-GAACATGTCTGCGTATCTC-3'; hsa-miR-1909-5p: 5'-TTGTGAGTGCCGGTGCCT-3', and reverse: 5'-GTGCAGGGTCCGAGGT-3'.

Functional enrichment analysis

We also evaluated each candidate miRNA targets which genes and also whether this is linked to GBM pathogenesis or not. To this end, bioinformatics analyses were performed using the Gene Ontology (GO) biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway options of the DIANA-miRPath v3.0²⁴. The pathway analysis was performed in order to determine which genes are targeted by the candidate miRNAs. We also used *miRTargetLink Humanto* to achieve comprehensive information on human miRNA-mRNA interactions²⁵. Furthermore, the miRWalk3.0²⁶ was utilized to find out each candidate miRNA targets which genes. We considered the miRNA-gene pairs that were in common in at least 5 databases.

To explore the clinical importance of the miRNA expression, we used the expression data of Gene Expression Omnibus (GEO) database²⁷. To this end, different data and several patients/controls were used and a comparison was performed between GBM and healthy samples. The following sources were used: hsa-let-7c-5p, source ID: GSE39486, and platform: GPL15829; hsa-miR-206-5p, source ID: GSE135189, and platform: GPL20906; and hsa-miR-1909-5p, source ID: SRP262521; In total, 54 GBM and 111 control samples were analyzed meticulously.

Identification of candidate prognostic biomarkers

GBM is notorious for poor prognosis²⁸, thus, finding miRNA-based biomarkers can be helpful to follow up the patients as effectively as possible²⁹. In this study, as GBM patients' follow-ups were impossible, we used Pan-Cancer Tool³⁰ to evaluate the association between the expression of each candidate miRNA and GBM prognosis. For each miRNA, we calculated hazard ratio, 95% confidence intervals (CI), and log-rank *P*-values.

Statistical analysis

We performed expression analyses using SPSS v.26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v.8.0 (GraphPad Software, Inc., La Jolla, CA, USA). The *student's t-test* was used and all experiments were repeated three times. To confirm the diagnostic value for each candidate miRNA, the receiver operating characteristic (ROC) curves were used, particularly by calculating the area under the curve (AUC) with at least 95% of confidence intervals (CI). *P*-values less than 0.05 were considered statistically significant.

Results

Patients and miRNA Expression analysis

We obtained a pair of 50 tumor and healthy tissue samples from GBM patients and healthy individuals, respectively. The patients' GBM was confirmed histopathologically. An overview of the patients' characteristics is put forward in **Supplementary Table 1**. The distribution of age and sex were matched between the two groups. Three miRNAs were selected and their expression was analyzed in a pair of 50 tumor and healthy tissue samples.

We used RT-qPCR to determine the expression levels of each candidate miRNA in the putative samples. To calculate the RT-qPCR efficiency, 5-fold serial dilutions of cDNA samples were prepared and standard curves were exploited. To this end, the slope of the standard curve was measured using this equation: $E = 10^{(-1/\text{slope})}$. The amplification efficiency of each miRNA and internal control was equal with a high linear correlation, highlighting the validation of the assay. Furthermore, we used a dissociation curve to underscore the uniqueness and specificity of the amplified products, i.e., the single and sharp peaks of the melting curves elucidated no primer-dimer or non-specific products.

The integrity of miRNAs that are derived from FFPE blocks has been proposed to be prone to modification and fragmentation in FFPE tissues³¹, however, due to the really small size of these molecules (about 19-22 nt), there is a little chance of degradation. Our results showed that hsa-let-7c-5p and hsa-miR-206-5p were downregulated in GBM samples, while hsa-miR-1909-5p showed an upregulation in tumor samples compared to healthy tissues (P -values < 0.0001) (Fig. 1a-c).

Correlation between miRNA expression and clinicopathological features

Any association between the expression of each miRNA and clinicopathological characteristics of GBM patients were analyzed. We investigated whether there is a relationship between the expression pattern of each miRNA and 'sex' and 'age' factors. We showed that except for hsa-let-7c-5p that was expressed abundantly in patients whose ages were < 50 years, the expression of hsa-miR-206-5p and hsa-miR-1909-5p did not show any association in terms of sex and age (Fig. 1d-i).

miRNA Biomarkers in GBM

To evaluate the sensitivity and specificity of each candidate miRNA, we analyzed the AUC-ROC curves that in turn showed that each miRNA potentially is a tumor and/or diagnostic biomarker for GBM. We considered the miRNAs as a diagnostic biomarker when their calculated AUC was above 0.50. The calculated AUC for hsa-let-7c-5p, -miR-206-5p, and -miR-1909-5p were 0.90, 0.80, and 0.81, respectively (P -values < 0.0001 , 95% of CI) (Fig. 2a-c).

Functional enrichment analysis

We used *miRTargetLink Human* to find the integrated network of the candidate miRNAs and their potential targets (Fig. 3a; **supplementary Fig. 1**). This resulted in the identification of several genes that were potential targets for hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p (Fig. 3a). Using DIANA-miRPath v.3.0, we also showed that the candidate miRNAs may contribute to different cell signaling procedures as in cell cycle, IGF signaling, and cell growth and proliferation (Fig. 3b). MiRWalk 2.0 was also used to confirm the identified possible interactions among the candidate miRNAs and their targets.

Candidate miRNA unsupervised hierarchical clustering analysis showed that the miRNAs discriminate GBM tumor and healthy tissues (Fig. 3c). GEO database was implemented to evaluate the expression levels of each candidate miRNA in GBM (Fig. 4a-c). The identified and predicted functions of each miRNA were determined also using the KEGG database (**Supplementary Fig. 1**).

Survival analysis of the miRNA-based signature

In this study, using *in silico* tools, we determined that the overall survival of GBM is low in cases with the aberrant expression of each candidate miRNA. The follow-up duration was determined at a maximum of 200 months (Fig. 4d-f). Our data showed that hsa-miR-206-5p may be a prognostic biomarker of GBM (HR

= 0.61). Regarding other miRNAs—i.e., hsa-let-7c-5p and hsa-miR-1909-5p—the potentiality of being a prognostic biomarker was not significantly detected (Fig. 4d-f).

Discussion

Despite some advances in surgical and adjuvant treatments, GBM patients still suffer from poor prognoses³². MiRNA-based biomarkers have some advantages to touch upon being easily obtainable in a non-invasive manner¹² and representing throughout the disease course and tumor status³³. Recently, miRNAs have been identified as promising noninvasive biomarkers that can show tumor development in early stages and so improves GBM diagnosis and prognosis³⁴. Herein, under the literature reviews and data mining using *in silico* tools, we chose three miRNAs including hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p that may contribute to GBM development. Different miRNAs have been detected that can successfully discriminate GBM, thus, we conducted an investigation into whether a signature of these candidate miRNAs can be a new diagnostic biomarker. To fill this yawning gap, the expression of each candidate miRNA was determined using RT-qPCR and their associations with clinicopathological features were analyzed.

Hsa-let-7c is a vital tumor suppressor miRNA playing a proactive role in the inhibition of proliferation, migration, and invasion of lung cancer cells³⁵; this miRNA is somehow linked to the etiology of recurrent lung cancer, although the exact mechanism yet still is blanketed in mystery. Recently, Luo *et al.* showed that hsa-let-7c-5p may regulate *CyclinD1* expression via the Wnt/ β -catenin signaling pathway in osteoblasts³⁶, suggesting that this miRNA may have a hand in cell cycling of tumor cells as well. Hsa-let-7c-5p is down-regulated in recurrent GBM than primary GBM samples³⁷. Also, hsa-let-7c inhibits metastasis in colorectal cancer and its down-regulation stimulates the expression of K-RAS, MMP11, and PBX3 that leads to the promoted cell migration and invasion^{38,39}. Therefore, hsa-let-7c-5p down-regulation may make a contribution to GBM recurrence via triggering GBM cell migration and invasion. Although there are many studies showing different roles of this miRNA in cellular and molecular events, its functions in GBM are still shrouded in mystery. Herein, we showed that this miRNA is downregulated in patients' samples than the control tissues and this was consistent with the data derived from GEO database (Fig. 4a-c); this verifies the tumor suppressor roles of this miRNA. The AUC-ROC curve analysis proposed that this miRNA is a reliable biomarker with good sensitivity and specificity that in turn is capable of distinguishing between GBM and healthy tissues, however, the prognostic potentiality was not detected *in silico*.

Zhang *et al.* showed that hsa-miR-206 suppressed cell proliferation in gastric cancer at least partially by targeting the *CyclinD2*⁴⁰. Similarly, the expression of this miRNA is downregulated in breast cancer⁴¹, laryngeal cancer⁴², rhabdomyosarcoma⁴³, colorectal cancer⁴⁴, and prostate cancer⁴⁵. These findings bestowed a tumor-suppressive role to hsa-miR-206-5p. This miRNA activates apoptosis and inhibits tumor cell migration⁴⁶. Overexpression of hsa-miR-206-5p gives rise to the decreased expression of metabolic genes, NADPH production, and ribose synthesis in mouse models⁴⁷. Furthermore, Hao *et al.*

showed that the expression of hsa-miR-206 was decreased in GBM tumor samples and pertinent cell models than in control tissues/cells⁴⁸. They demonstrated that hsa-miR-206 is a tumor suppressor and its inhibition sets stages for GBM development and progression. Our findings were in line with these results. Using AUC-ROC curve analysis, we showed that hsa-miR-206-5p is a promising biomarker that can discriminate the GBM from healthy tissues. Also, using *in silico* data, we showed that this miRNA is a probable prognostic biomarker for GBM, however, further studies are required in order to show the exact molecular mechanisms whereby this miRNA functions in GBM development/progression.

Hsa-miR-1909-5p is up-regulated in pediatric dysembryoplastic neuroepithelial tumors, casting light on its oncogenic functions⁴⁹. Braoudaki *et al.* showed that this miRNA is a potential biomarker distinguishing between the tumor types and the normal tissues⁴⁹. Furthermore, it has been identified that hsa-miR-1909-5p was significantly up-regulated in less aggressive rectal cancer patients⁵⁰. Hsa-miR-1909 is expressed in the early stages of gastric cancer⁵¹, however, regarding GBM, there is a snippet of information about the roles of this miRNA in the development or progression; for instance, Li *et al.* elucidated that miR-1909-5p was significantly upregulated in a GBM patient⁵². Using a high-throughput technique, it has been identified that miR-1909-5p was upregulated in GBM tumors⁵³. Our results underscored that miR-1909-5p functions oncogenic roles in GBM development, however, further studies are needed to arrive at a conclusion and to unearth the detailed underlying molecular mechanisms. On the other side, it has been recently demonstrated that miR-1909-5p may play a tumor-suppressive role in the etiology of Parkinson's disease, i.e., its downregulation was identified in postmortem samples of patients with Parkinson's disease⁵⁴. This study can per se show what complications are expectable when it comes to miRNAs. In a nutshell, we demonstrated that hsa-miR-1909-5p is a diagnostic biomarker in GBM and is upregulated in GBM patients, proved by *in silico* tools. Long term follow-ups did not show any prognostic biomarker potentiality for this miRNA.

In this study, we also showed that hsa-let-7c-5p, hsa-miR-206-5p, and hsa-miR-1909-5p target different genes that most of which are involved in cell cycle, IGF signaling, cell proliferation/growth, and cell migration. This may substantiate that the signature of these miRNA may represent different cellular processes that are often dysregulated in GBM.

There are some limitations in this study; for example, as obtaining direct samples from GBM tumors needs surgery, it would be feasible and more convenient if we did check the expression of the signature in serum, plasma, or cerebrospinal fluids. Therefore, further studies seem to be imperative to determine the exosomal levels of this signature to show whether this can be used as a non-invasive method to discriminate tumor and healthy samples or not. Likewise, as *in silico* analysis showed, the signature targets a substantial number of genes; so, miRNA and gene expression profiling is necessary to determine which genes are targeted by the signature. Thirdly, to confirm our findings, conducting larger investigations with more diverse samples would be helpful.

Conclusions

In the present study, we shed light on this notion that a signature of three miRNAs (i.e., hsa-let-7c-5p, -206-5p, and -1909-5p) is capable of distinguishing GBM tumor and healthy tissues; however, further studies are essential to unveil the detailed molecular mechanisms whereby these miRNAs play roles in GBM development.

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Figures

Figure 1

The expression analysis of the candidate miRNAs in GBM and healthy tissues. a) hsa-let-7c-5p is downregulated in GBM patients compared to the control group, b) hsa-miR-206-5p is downregulated in tumor samples, c) hsa-miR-1909-5p is upregulated in tumor tissues compared to the healthy tissues. d-f) there are no associations between the expression of each candidate miRNA and the participants' sex. g) the expression of hsa-let-7c-5p is correlated with the 'age' factor, i.e., higher expression of this miRNA was

observed in patients who were < 50 years. h and i) no association was detected between the expression of hsa-miR-206-5p and -1909-5p and age factor. In this figure, ** P-value < 0.01, **** P-value < 0.0001, and ns: not significant.

Figure 2

ROC curve analysis was performed for each candidate miRNA. Vertical axes represent the sensitivity, while the horizontal ones show specificity. The area under the curve (AUC) was used to measure the diagnostic performances for each candidate miRNA. We considered miRNA as a biomarker if its calculated AUC was above 0.5. The AUC scores calculated for hsa-let-7c-5p(a), -miR-206-5p(b), and -miR-1909-5p (c) were 0.90, 0.80, and 0.81 (P-values < 0.0001), respectively.

Figure 3

Functional enrichment analysis. a) miRNA-mRNA interactions were obtained using miRTargetLink Human. All findings were also confirmed using miRWalk 2.0. b) A miRNA vs. GOSlim groups heatmap was drawn using DIANA-miRPath v3.0 that in turn show the enrichment level in GO categories of the candidate miRNAs. c) Unsupervised hierarchical clustering analysis confirmed that each miRNA is differentially expressed in GBM compared to healthy tissues. The heatmaps were obtained using the GEO database.

Figure 4

Expression and prognostic values of each miRNA were calculated and confirmed using in silico tools. the expression of each candidate miRNA was determined using the GEO database, a) hsa-let-7c-5p, b) hsa-miR-206-5p, and c) hsa-miR-1909-5p. The GBM dataset from Kaplan Meier Plotter was used to test for the survival prediction capacity of each candidate miRNA. Samples were divided into two distinct groups including Low (blue) and High (red) expression groups for each target. In total, at least 500 individuals were analyzed. Hazard ratio (HR) and P-value for each association are shown within each plot. In this figure, d) hsa-let-7c-5p, e) hsa-miR-206-5p, and f) hsa-miR-1909-5p. P-values < 0.05 were considered as valid data.

Supplementary Files

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