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Bioinformatics analysis of miRNA expression profile between primary and recurrent glioblastoma

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Abstract. – OBJECTIVE: Glioblastoma (GBM) is the most malignant brain tumor with rapid relapse. The goal of this study is to identify microRNAs (miRNAs) involved in recurrent GBM.

MATERIALS AND METHODS: miRNA transcription profile data (GSE32466) were downloaded, including 12 primary GBM samples and 12 recurrent GBM samples. Then, limma package was utilized to identify differentially expressed miRNAs (DEMs) with the criteria of false discovery rate < 0.05 and |log2 fold change| ≥ 1. Thereafter, miTarget and TargetScan databases were used to predict the potential target genes of DEMs. Regulatory co-expression network was constructed based on co-expressed genes and potential miRNA-gene pairs, and then, pathway analysis was conducted. Furthermore, database miRWalk was used to screen out known GBM-associated miRNAs from the identified DEMs.

RESULTS: A total of 71 DEMs were identified between primary and recurrent GBM samples, and 2684 potential target genes were found. Besides, regulatory co-expression network was constructed, including 12 DEMs and 81 potential target genes. These genes significantly enriched in ECM-receptor interaction, ribosome, and focal adhesion pathways, and DEMs like hsa-miR-320a, hsa-miR-139-5p, has-miR-128, hsa-miR-140-5p, and hsa-miR-146b-5p had high degree. Notably, 7 DEMs in network were known GBM-associated miRNAs recorded in database miRWalk.

CONCLUSIONS: DEMs like hsa-miR-320a, hsa-miR-139-5p, has-miR-128, hsa-miR-146b-5p, hsa-let-7c, hsa-miR-128, and hsa-let-7a might participate in recurrent GBM. These results would pave ways for further study of recurrent GBM mechanism, and for the prevention and treatment of recurrent GBM. However, more experimental verifications are required to prove these predictions.

Key Words:
Co-expressed analysis, Differentially expressed miRNA, Pathway analysis, Recurrent glioblastoma, Target gene.

Introduction

Glioblastoma (GBM), also known as Grade IV astrocytoma, is the most deadly malignant type of brain tumor that arises from glial cells or their precursors. GBM affects 2-3 of 100,000 persons per year, and it accounts for 12-15% of all brain tumors and approximately 70% of all diagnosed gliomas[1]. GBM usually recurs independently of the types of treatment like surgical resection followed by radiation or chemotherapy[2]. At the time of GBM recurrence, few treatment options are available, leading to a median survival of approximately 6 months[3,4]. Recently, bevacizumab, which is a monoclonal antibody against vascular endothelial growth factor (VEGF), has been reported to possess clinically meaningful activity for recurrent GBM[5]. Although many efforts have been made, no improvement is observed in the overall survival rate of recurrent GBM. Hence, novel target molecules are needed for the treatment of recurrent GBM.

In recent years, several molecular biomarkers for primary GBM have been identified, including 1p/19q co-deletion, hypermethylation of the methyl guanine methyl transferase (MGMT) promoter, gene mutations in important genes (isocitrate dehydrogenase 1 (IDH1), epidermal growth factor receptor (EGFR), and p53), and dysregulation of microRNAs (miRNAs)[6,7]. However, the molecular mechanism associated with GBM recurrence remains still elusive. Martinez et al[8] have illustrated that the genetic profiles of type 1 GBM and type 2 GBM are conserved at the time of relapse, and PTEN mutations and loss of heterozygosity were detected upon relapse in type 2 GBM. Besides, hypermethylation of CASP8 is a frequent feature of relapsed GBM in comparison with the corresponding primary tumor[9]. Notably,

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cancer stem cells (CSCs) have been suggested as the reason of tumor recurrence, as CSCs can stimulate tumor angiogenesis by expressing elevated levels of VEGF and CSCs possess aggressive and invasive properties[10]. As a family of small non-coding RNAs, miRNAs play vital regulatory roles in diseases via regulating expression of protein-coding target genes at both post-transcriptional and translational levels. However, few studies have been conducted to investigate the miRNAs in recurrent GBM.

In order to further study recurrent GBM mechanism in miRNA aspect and, thus, find novel therapeutic targets, we firstly downloaded miRNA expression profiles of primary and recurrent GBM samples and, then, analyzed these data to identify the differentially expressed miRNAs (DEMs) between primary and recurrent GBM samples. Subsequently, target genes of DEMs were identified, and the co-expression relationships among target genes were analyzed, forming regulatory co-expression network. Furthermore, convinced miRNAs were identified by comparing DEMs with previous studies. This study identified potential miRNAs and the corresponding potential target genes involved in recurrent GBM, providing new directions for the mechanism studies on GBM, as well as prevention and treatment of recurrent GBM.

Materials and Methods

miRNA Expression Profiles

The miRNA expression profiles of primary GBM (PGBM) samples and recurrent GBM (RGBM) samples were obtained from Gene Expression Omnibus (GEO) database[11]. The access number is GSE32466 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32466), and the dataset has a total of 24 samples, including 12 PGBM samples and 12 RGBM samples. The platform used here was GPL10850Agilent-021827 Human miRNA Microarray (V3) (miRBase release 12.0 miRNA ID version).

Identification of DEMs

The probe-level data were first converted into expression values. For each sample, the expression values of all probes for a given gene were averaged to obtain a single expression value. Then, we imputed missing data and performed quartile data normalization using robust multi-chip average (RMA) method[12,13]. Finally, the limma package[14] in R language was employed to identify DEMs between PGBM samples and RGBM samples. The *p*-value was then adjusted by using Benjamini-Hochberg method[15], generating false discovery rate (FDR). We defined FDR < 0.05 and $|\log_2$ fold change (FC)$| \geq 1$ as the threshold for this analysis.

Hierarchical Clustering Analysis of DEMs

In order to show the sample-specificity of DEMs, hierarchical clustering[16] was conducted and Euclidean distance[17] was calculated by utilizing Pheatmap package[18] in R language.

Target gene prediction of DEMs

Identification of gene targets is critical for characterizing the functions of miRNAs. Reportedly, miTarget is a reliable tool for predicting target genes of miRNA based on support vector machine classifier[19]. Based on thermodynamics-based modeling of RNA duplex and comparative sequence analysis, TargetScan can predict miRNA target genes conserved across multiple genomes[20]. In this study, miTarget[19] and TargetScan[20] databases were used to predict the target genes of DEMs. To make our predicted target genes more convinced, only the common target genes were selected, which were defined as potential target genes.

Construction of the Regulatory co-expression Network

An essential prerequisite for understanding cellular functions at molecular level is to correctly uncover all functional interactions among various proteins. As functional interactions generally require co-appearance of proteins, co-expression analysis is useful for identifying novel genes/proteins in biological functions. The Search Tool for the Retrieval of Interacting Genes (STRING)[21] is an online tool that includes plenty of co-expression relationships validated by experiments. In this study, potential target genes with co-expression coefficients higher than 0.6 were extracted from STRING. Thereafter, Cytoscape software[22] was used to visualize the regulatory co-expression network by using these co-expression relationships and potential miRNA-target pairs.

Pathway Analysis of Regulatory co-expression Network

Kyoto encyclopedia of genes and genomes Orthology-based Annotation System (KOBAS)[23] can provide the most comprehen-

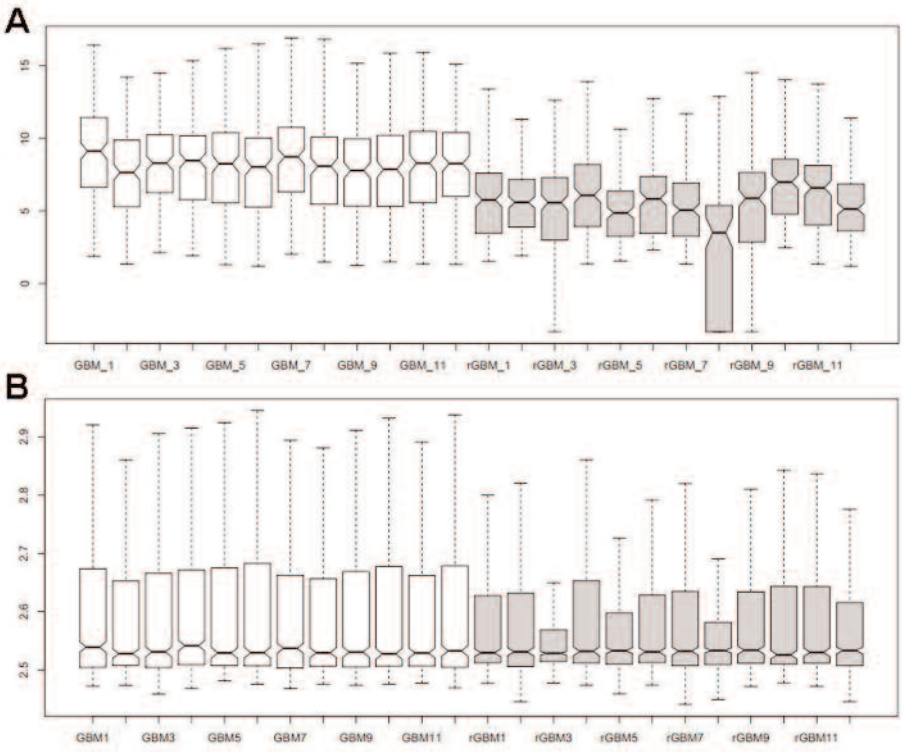

Figure 1. microRNA expression profiles of primary and recurrent glioblastoma. ***A,*** Expression profiles before normalization. ***B,*** Expression profiles after normalization. White columns represent primary glioblastoma samples. Grey columns represent recurrent glioblastoma samples.

sive set of functionalities and find statistically enriched pathways. Here, KOBAS was used to identify the pathways that involve potential target genes in network with a criterion of p-value < 0.05.

Comparison with Previous Studies

Reportedly, miRWalk[24] is a comprehensive database that provides information on miRNAs of human, mouse, and rat. Furthermore, it also provides information on experimentally validated miRNA interaction, associated genes, pathways, disease, cell lines, organs, and literatures. In the present study, we used the Validated Targets module of miRWalk to compare miRNAs identified here with the previously identified GBM-associated miRNAs.

Results

Identification of DEMs and Hierarchical Clustering Analysis

After preprocessing, standard miRNA expression profiles were obtained (Figure 1). A total of 71 DEMs were identified (FDR < 0.05 and $|\log_2 FC| \geq 1$), including 44 up-regulated DEMs and 27 down-regulated DEMs. Thereafter, hierarchical clustering analysis illustrated that DEMs could distinguish PGBM and RGBM samples apparently (Figure 2).

Target Prediction of DEMs and Construction of Regulatory co-expression Network

Database miTarget and TargetScan were used to predict the target genes of DEMs, and the common set of these genes were selected, generating 2684 potential target genes (Figure 3). Then, a total of 103 validated co-expressed gene pairs were identified based on STRING. After combining these gene pairs with potential miRNA-target pairs, regulatory co-expression network was constructed, which was visualized via Cytoscape (Figure 4). This network contained 93 nodes including 12 DEMs and 81 potential target genes (Table I). Several DEMs had high degrees, like has-miR-320a, hsa-miR-139-5p, has-miR-128, hsa-miR-140-5p, and has-miR-146b-5p (Figure 4).

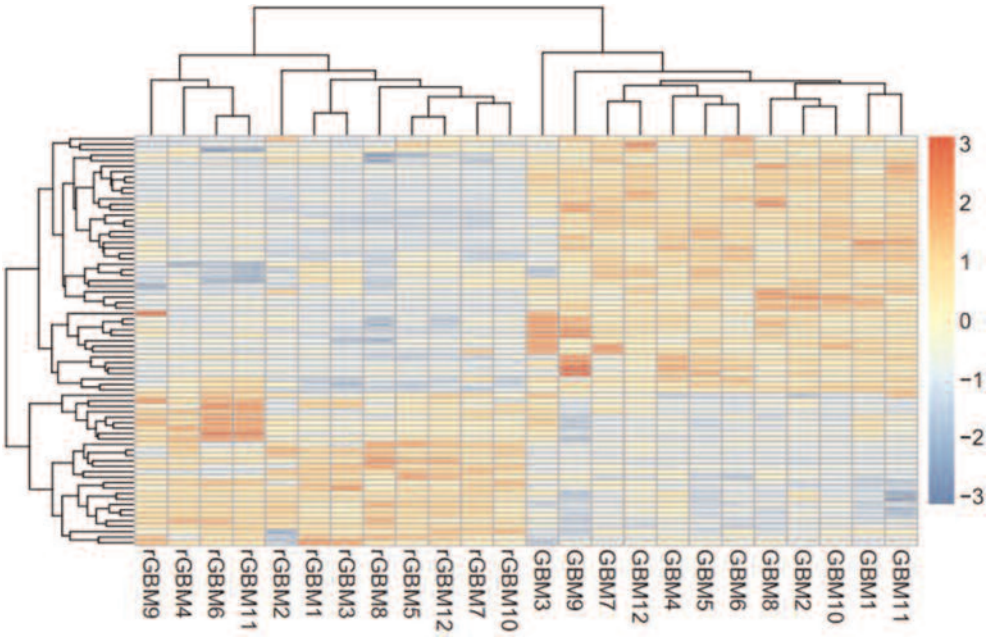

Figure 2. Hierarchical Clustering analysis of differentially expressed microRNAs. Red represents high expression value, while blue represents low expression value. GBM: glioblastoma; rGBM: recurrent glioblastoma.

Pathway Analysis of Regulatory co-expression Network

KOBAS was used to perform pathway analysis, and 3 pathways were identified to involve the potential target genes in regulatory co-expression network (Table II). Notably, ECM-receptor interaction was the most significant pathway, and it involved three genes *COL4A1*, *COL3A1* and *COL1A2* targeted by has-let-7a, has-miR-128 and has-let-7c, respectively.

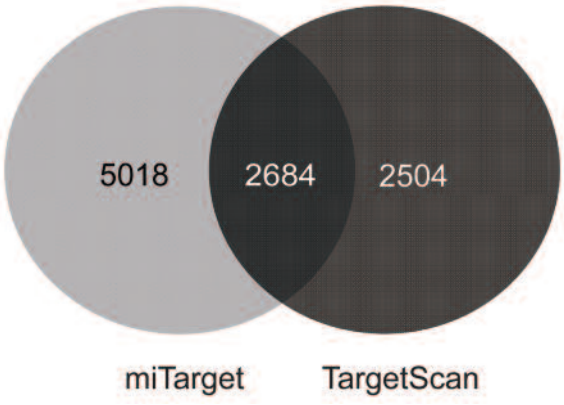

Figure 3. Target genes predicted based on miTarget database and TargetScan database.

Comparison with Previous Studies

A total of 75 miRNAs directly related with GBM were extracted from miRWalk database. After comparing these miRNAs with DEMs in the regulatory co-expression network, 7 DEMs were known GBM-related miRNAs validated in previous studies, including hsa-let-7a, hsa-let-7c, hsa-miR-128, hsa-miR-140-5p, hsa-miR-153, hsa-miR-219-5p, and hsa-miR-486-5p (Table I).

Discussion

GBM is an extremely infiltrative tumor of which 95% recur within 2 cm of primary tumor[1]. Treatment of recurrent GBM is still unsolved. In this study, bioinformatics analyses were performed to investigate the potential molecular mechanism of recurrent GBM and identify molecular targets at the aspect of miRNA.

Regulatory co-expression network was constructed, and it involved 12 DEMs, including hsa-miR-320a, hsa-miR-139-5p, has-miR-128, hsa-miR-140-5p, and hsa-miR-146b-5p with high degree. Among these DEMs, has-miR-128 and hsa-miR-140-5p have been validated to participate in GBM, while hsa-miR-320a, hsa-miR-139-5p, and hsa-miR-146b-5p have not. Reportedly, miR-320a is dysregulated in GBM patients,

Table I. Differentially expressed miRNAs in regulatory co-expression network.

miRNA	Degree	miRNA	Degree
has-miR-320a	23	**hsa-miR-486-5p**	8
hsa-miR-139-5p	14	**hsa-miR-153**	5
has-miR-128	12	**hsa-miR-219-5p**	4
hsa-miR-140-5p	12	hsa-miR-338-3p	3
has-miR-146b-5p	11	hsa-miR-142-3p	3
has-let-7c	9	**hsa-let-7a**	2

miRNAs in bold represent the glioblastoma-related miRNAs validated in previous studies. miRNAs: microRNAs.

and miR-320a overexpression can inhibit cell proliferation, migration, invasion, and tumorigenesis by targeting insulin-like growth factor-1 receptor (IGF-1R)[25]. Notably, Lim et al[26] have illustrated that miR-320a can predict the recurrence of colorectal cancer, and it participates in biological functions like cell growth and survival, tumour-stromal cross-talk, and angiogenesis.

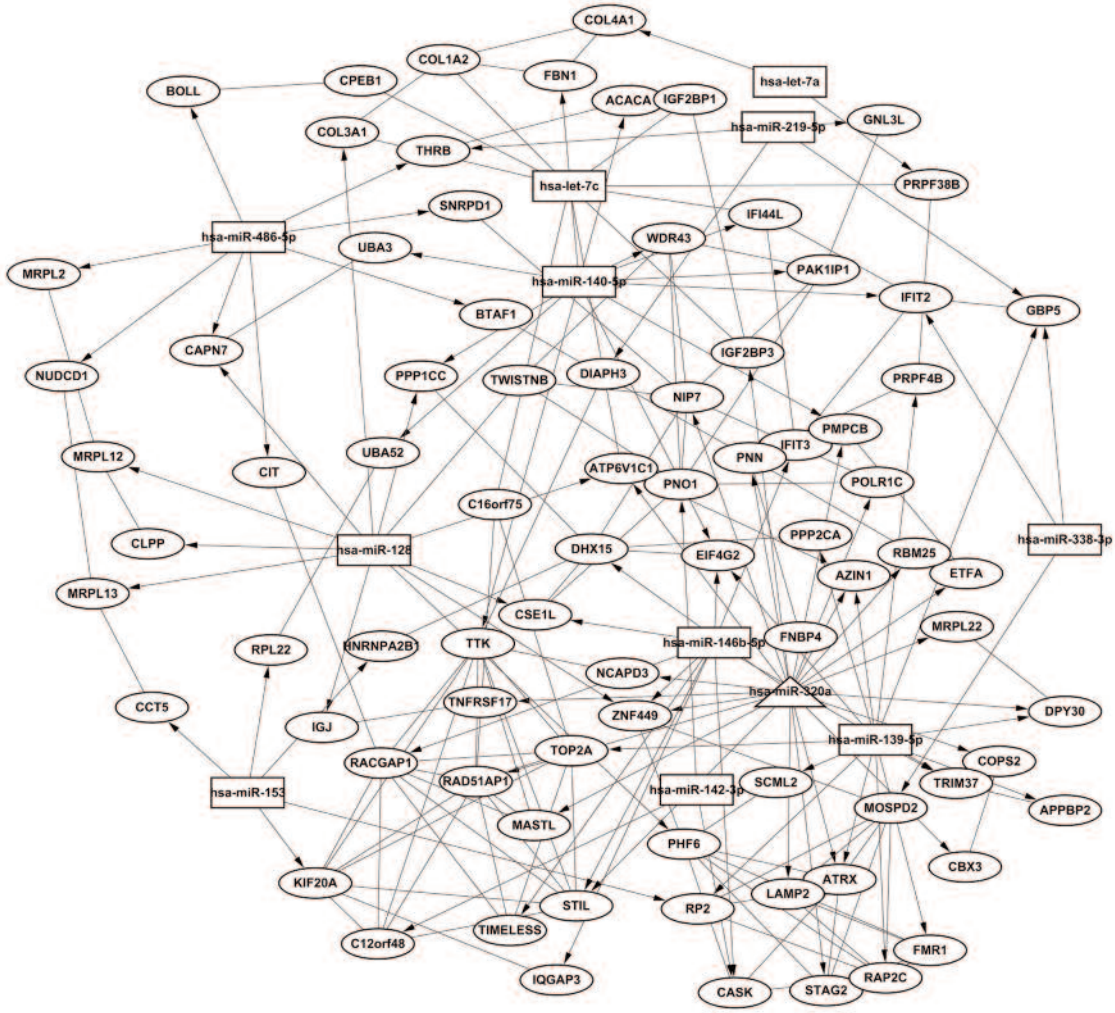

Figure 4. Regulatory co-expression network of DEMs and potential target genes. Squares represent down-regulated DEMs. Triangles represent up-regulated DEMs. Circles represent potential target genes. DEMs: differentially expressed microRNAs between primary and recurrent glioblastoma samples.

Table II. KEGG pathway analysis of potential target genes in regulatory co-expression network.

ID	Pathway	*p*-value	Potential target genes
hsa04512	ECM-receptor interaction	0.00679	*COL4A1, COL3A1, COL1A2*
hsa03010	Ribosome	0.01216	*MRPL13, RPL22, UBA52*
hsa04510	Focal adhesion	0.02122	*COL4A1, COL3A1, COL1A2, PPP1CC*

KEGG: Kyoto encyclopedia of genes and genomes; ID: identifier; ECM: extracellular matrix.

Therefore, miR-320a might play a role in recurrent GBM. Moreover, Wong et al[27] have found that down-regulation of miR-139 can increase the invasive abilities of hepatocellular carcinoma (HCC) cells *in vitro* and HCC metastasis *in vivo*. Actually, miR-139-5p is significantly correlated with matrix metalloproteinase-9 (MMP-9) expression, which can promote the invasion of glioma cells[28]. In this study, miR-139-5p was down-regulated in RGBM samples compared with PGBM samples, indicating that its down-regulation might promote GMB recurrence. Furthermore, miR-146b-5p is located within 10q24-26, a chromosomal region most frequently missing in GBM[29]. Our result showed that miR-146b-5p is down-regulated in RGBM samples compared with PGBM samples. Lin et al[30] has found that overexpression of miR-146b-5p could significantly reduce the migration and invasion of pancreatic cancer stem cells and, therefore, induce cancer recurrence. Katakowski et al[31] have illustrated that miR-146b-5p suppresses EGFR translation by binding to its 3'-untranslated region, and EGFR is essential for CSCs maintenance[32]. Therefore, miR-146b-5p down-regulation promotes the maintenance of CSCs, revealing that miR-146b-5p down-regulation may induce GBM recurrence. Altogether, miR-128, 140-5p, miR-320a, miR-139-5p, and miR-146b-5p might participate in recurrent GBM.

Pathway analysis showed that target genes in network significantly enriched in ECM-receptor interaction and focal adhesion pathways, and the related genes were targeted by hsa-let-7c, hsa-miR-128 and hsa-let-7a. Tumor dormancy refers to that cancers may recur either locally or distantly (metastasis) many years after apparently successful primary treatment[33]. Extracellular matrix (ECM) may play an important role in regulating maintenance of dormancy and release from dormancy, and focal adhesions physically connect extracellular matrix (ECM) to cytoskeleton and mediate cell migration[34,35]. Our results showed that miR-128 is down-regulated in RGBM samples compared with PGBM samples. Reportedly, miR-128 is significantly correlated with matrix metallopeptidase-9 (MMP-9) expression in GBM[28]. MMP-9 belongs to the zinc-metalloproteinase family involved in the degradation of ECM, and *MMP-9* is an important oncogene that improves invasiveness of cancer cell which is essential for metastasis and recurrence[36]. Additionally, miR-128 is downregulated in GBM and lower grade gliomas, and its expression can significantly reduce glioma cell proliferation and glioma xenograft growth[37]. Especially, miR-128 can inhibit brain cell proliferation by targeting E2F3α[38], and block glioma self-renewal by targeting oncogene BMI-1[37]. Therefore, miR-128 down-regulation might promote cancer invasion and GBM recurrence. In addition, our results showed that let-7c is down-regulated in RGBM samples compared with PGBM samples. Han et al[39] has suggested that let-7c is a metastasis suppressor in colorectal cancer, as let-7c down-regulation promotes the expression of K-RAS, MMP11 and PBX3 and, thus, promotes cell migration and invasion. Therefore, let-7c down-regulation might participate in GBM recurrence via promoting cancer cell migration and invasion. In addition, increased let-7a expression contributes to increased cell proliferation and ECM deposition[40,41]. Therefore, hsa-miR-128, hsa-let-7c, and hsa-let-7a might regulate the recurrence of GBM.

Conclusions

hsa-miR-320a, hsa-miR-139-5p, hsa-miR-146b-5p might play roles in recurrent GBM, while hsa-let-7c, hsa-miR-128, and hsa-let-7a might regulate GBM recurrence through ECM-receptor interaction and focal adhesion. Although more experimental verifications are still needed to prove these predictions, our results might direct further study of recurrent GBM mechanism and the prevention and treatment of recurrent GBM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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