Downregulation of microRNA-374a predicts poor prognosis in human glioma

QIANG DONG[1*], GUOQIANG YUAN[2*], MIN LIU[3*], QIQI XIE[4], JIANHONG HU[1],
MAOLIN WANG[1], SHANGYU LIU[1], XIAOJUN MA[1] and YAWEN PAN[1,2]

[1]Department of Neurosurgery, The Second Hospital of Lanzhou University;
[2]Institute of Neurology, The Second Hospital of Lanzhou University, Lanzhou, Gansu 730030;
[3]Department of Pharmacy, Hebei North University, Zhangjiakou, Hebei 075000; [4]Department of Orthopaedics,
The Second Hospital of Lanzhou University, Lanzhou, Gansu 730030, P.R. China

Abstract. Certain microRNAs (miRNAs/miRs) may be used as prognostic biomarkers in various types of cancer. The purpose of the present study was to identify miRNAs that were abnormally expressed in glioma of different grades, and to evaluate their clinical implications in patients with glioma. The differentially expressed miRNAs were evaluated from the expression profiles of six glioma tissues (three low-grade and three high-grade gliomas) determined using a microarray platform. Reverse transcription-quantitative polymerase chain reaction analysis was used to further verify the aberrant expression of the candidate miRNA in a set of 42 patients and 5 healthy controls. The miRNA target genes were predicted and the protein-protein interaction network was generated; furthermore, functional enrichment analysis of the target genes in Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed. Kaplan-Meier curves and Log-rank analysis, as well as multivariate Cox regression analysis were performed to assess the association of the candidate miRNA with patient survival. A total of 15 differentially expressed miRNAs, including 13 downregulated and 2 upregulated miRNAs, were identified by comparison of low-grade and high-grade glioma tissues. The miR-374a expression of high-grade gliomas was significantly lower than that of low-grade gliomas (fold change, -4.43; P=0.027). The expression levels of miR-374a gradually decreased with the increase of the pathological grade of glioma. Pearson's Chi-square test was used to determine the association of miR-374a expression with several clinicopathological factors. Furthermore, low expression of miR-374a was determined to be an independent prognostic marker and that it was significantly associated with overall survival (P=0.0213). GO and KEGG pathway analysis revealed that the target genes of miR-374a may be involved in the regulation of the RNA polymerase II promoter and mTOR signaling pathway. The four hub genes (*CCND1, SP1, CDK4, CDK6*) were also identified by PPI network analysis. In conclusion, the present study indicated that miR-374a may be used as a promising prognostic biomarker for the screening of high-risk populations and for the assessment of the prognosis of patients with glioma.

Introduction

Glioma is the most common type of primary brain tumor in adults, accounting for ~46% of intracranial tumors, with an incidence rate of 3-10/10 million, corresponding to 1-3% of detected malignancies worldwide (1,2). Grade-I and -II gliomas are classified as benign and low-grade gliomas, while grade-III and -IV gliomas are classified as high-grade gliomas, according to the World Health Organization (WHO) grading system (3). The current standard treatment for gliomas mainly includes surgery, followed by radiotherapy and chemotherapy. However, the therapeutic effect is not satisfactory. The median survival time of glioblastoma multiforme (GBM; grade 4 astrocytoma) patients remains poor, ranging from 12 to 15 months, 2 to 5 years and 6 to 8 years for grade IV, III and I-II gliomas, respectively according to the WHO grading system (4) Previous studies have proven that cytogenetic and molecular analyses have a vital role in predicting the remission and survival rates of patients. The mutation status of isocitrate dehydrogenase, NADP+, the heterozygous deletion at the chromosomal position 1p/19q and the methylation of the O-6-methylguanine-DNA methyltransferase promoter (5-7) have been progressively used as diagnostic and prognostic markers for the comprehensive assessment of glioma patients. However, biological differences between individual patients are apparent and novel biomarkers are required to fully evaluate the prognosis of patients and predict the effectiveness of glioma treatment.

Correspondence to: Professor Yawen Pan, Department of Neurosurgery, The Second Hospital of Lanzhou University, 82 Cuiyingmen Road, Chengguan, Lanzhou, Gansu 730030, P.R. China
E-mail: panyw2018@163.com

*Contributed equally

MicroRNAs (miRNAs/miRs) are a class of short endogenous non-coding RNA molecules that are 18 to 25 nucleotides in length and exert a tumor-regulatory function at the post-transcriptional level by binding to the 3'-untranlated region (3'-UTR) and frequently to the 5'-UTR of mRNA molecules. Accumulating studies have identified multiple dysregulated miRNAs, which may serve as key molecules in cancer progression, and regulate various biological processes, including proliferation, differentiation, apoptosis and survival (8-10). Previous studies have demonstrated a significant difference in the expression profile of miRNAs between healthy subjects and patients with glioma, suggesting that the expression levels of certain miRNAs are associated with the overall survival (OS) of glioma patients (11-14). Therefore, the identification of differentially expressed miRNAs is of great importance in order to evaluate the early prognosis of glioma patients.

In the present study, a microarray-based analysis was performed to recognize differentially expressed miRNAs in gliomas by comparing miRNA expression profiles among low-grade and high-grade gliomas. Furthermore, the differential expression of miR-374a in human glioma tissues was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The prognostic value of this differentially expressed miRNA was then investigated. Furthermore, a bioinformatics analysis based on miRNA target gene databases was used to identify target genes of miR-374a. The GO and KEGG analysis were performed and the hub genes were analyzed by construction of a protein-protein interaction (PPI) network for the target genes. The aim of the present study was to obtain novel prognostic and predictive biomarkers for glioma, and to investigate the potential mechanisms of glioma progression.

Patients and methods

Patients and tissue specimens. Glioma tissues (15 grade II, 13 grade III and 20 grade IV) from a total of 48 patients were collected from the Department of Neurosurgery of the Second Hospital of the Lanzhou University (Gansu, China), from January 2013 to December 2016. The 5 normal brain tissues from patients without glioma who underwent surgery for other reasons, including cerebral trauma. Following surgical removal, the tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until used for RNA isolation. Patients who had received chemotherapy or radiotherapy prior to surgery were excluded. During the follow-up, OS was observed from the date of diagnosis to the date of patient death and/or the last census date in case of the patient being alive.

Microarray. A total of six glioma tissues (3 low-grade and 3 high-grade gliomas) were next analyzed using microarray methodologies in order to screen for the expression of miRNAs. A total of 1 μg of total RNA was extracted from each tumor sample using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and first-strand complementary (c)DNA synthesis was performed using the miRNA First-Strand cDNA Synthesis kit (cat. no. AS-MR-004; Arraystar, Rockville, MD, USA) following the manufacturer's protocols. The miRStar™ Human Cancer Focus miRNA

PCR Array (cat. no. AS-MR-0033; Arraystar) was applied on the ABI PRISM7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each 384-well miRStar™ Human Cancer Focus miRNA PCR Array contained 184 miRNAs linked to human cancers, nine wells for different housekeeping miRNAs, a genomic DNA contamination control, three replicate RT controls and three replicate positive PCR controls. The values of the quantification cycle (Cq) that were obtained for quantification were used for the calculation of fold changes in miRNA abundance according to the $2^{-\Delta\Delta Cq}$ method (15). The raw data were processed by the following workflow: Background detection, RMA global background correlation, quantile normalization, median polish adjustment was performed and log2-transformation with miRNA QC tool software (Affymetrix; Thermo Fisher Scientific, Inc.) (16).

RT-qPCR. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNA was randomly synthesized from 2 μg total RNA using the miRcute Plus miRNA First-Strand cDNA Synthesis Kit (cat. no. KR211; Tiangen Biochemical Technology Beijing Co. Ltd., Beijing, China) according to the manufacturer's instructions. RT products were amplified using SYBR Green PCR (cat. no. FP411; Tiangen Biochemical Technology Beijing Co. Ltd.) on a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was performed according to the manufacturer's instructions. The relative mRNA expression data were acquired and analyzed using the $2^{-\Delta\Delta Cq}$ method, and the expression values were normalized to U6, which was used as an internal control. The primers used in the present study were as follows: miR-374a forward, 5'-CGGCGGTTATAA TACAACCTG-3' and reverse, 5'-AGCTCGAGTGGAAGT CTGTGCA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACAT ATA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Data analysis. The median value of the expression levels of miR-374a was used as the cut-off point, and the patients were divided into a high-level and a low-level group. The expression levels of miR-374a between glioma and normal brain tissues were compared with the independent-samples t-test. Pearson's Chi-square test was used to analyze the association between miR-374a expression levels and the clinicopathological characteristics of the patients. The OS rates of the patients in the high-level and low-level groups were evaluated using the Kaplan-Meier method. Furthermore, univariate and multivariate Cox proportional hazard regression models were used to evaluate the prognostic value of multiple variables, including miR-374a expression, sex, age and Karnofsky performance status (KPS) score.

Bioinformatics analysis. The target genes of miR-374a were predicted using TargetScan (http://www.targetscan. org/) (17), miRTarBase (http://mirtarbase.mbc.nctu.edu. tw/php/search. php) (18), starBase (http://starbase.sysu. edu.cn/browseIntersectTargetSite.php) (19) and miRDB (http://www. mirdb.org/) (20). To enhance the reliability of the bioinformatics analysis, the overlapping target genes were identified using a Venn diagram. To further investigate the functions of these consensus target genes, the Database for
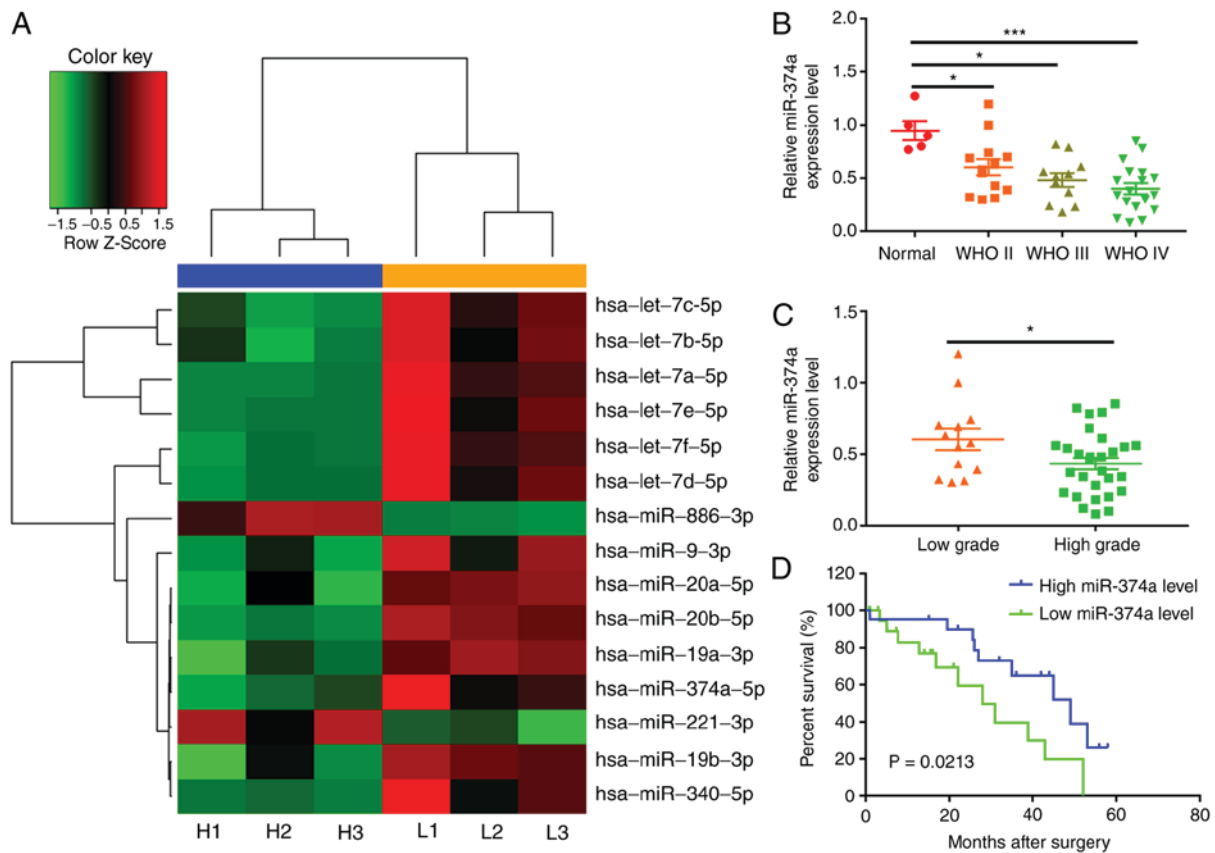
Figure 1. (A) Heatmap of the differentially expressed miRNAs between low-grade gliomas (n=3) and high-grade gliomas (n=3). Each row represents the expression levels of a specific miRNA and each column represents a single sample: Orange color denotes low-grade gliomas, whereas blue denotes high-grade gliomas. (B) The relative expression levels of miR-374a in glioma samples of different grades (n=42) were significantly decreased compared with those in normal tissues (n=5); furthermore, the expression of miR-374a slightly increased in glioma with increasing grade. (C) miR-374a expression levels in high-grade glioma tissues (n=13) and low-grade glioma tissues (n=29; P<0.05). *P<0.05, ***P<0.001. (D) Kaplan-Meier curves for overall survival in patients with gliomas stratified according to high/low expression of miR-374a. miRNA/miR, microRNA; hsa, Homo sapiens; H, high-grade gliomas; L, low-grade gliomas; WHO, World Health Organization.

Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool (https://david.ncifcrf.gov/) and mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/) were used to perform gene ontology (GO) and Kyoto Encyclopedia of genes and genomes (KEGG) pathway enrichment analyses. These analyses aimed to predict protein interactions, which included physical and functional associations. The present study used Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/) to construct the PPI network for target genes (minimum required interaction score. >0.4). In addition, Cytoscape software version 3.6.0 (http://www.cytoscape.org/download-platforms.html) was used for visualization of the PPI networks.

Statistical analysis. All analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Data calculations from each experiment were performed independently at least three times and values are expressed as the mean ± standard error of the mean. Differences between groups were assessed by one-way analysis of variance followed by an LSD post-hoc test and a Student's t-test. The Kaplan-Meier method was used to estimate the OS curves. P<0.05 was considered to indicate statistical significance.

Results

miRNA profiling. The miRNA profile was analyzed for low-grade (n=3) and high-grade gliomas (n=3) using the miRStar™ Human Cancer Focus miRNA PCR Array, which allowed for the assessment of the expression levels of 184 miRNAs associated with cancer. The median expression levels of each miRNA in each of the two groups were calculated, and the differences between them were determined using the t-test. P<0.05 was considered to indicate significant differences in expression and a fold change of >2 was set as the cut-off value. A total of 15 miRNAs, including 13 downregulated and 2 upregulated miRNAs, were identified between low-grade and high-grade glioma tissues (Fig. 1A; Table I). As the differences in multiples of miR-374a were the largest, miR-374a was selected for further study. It was revealed that the expression levels of miR-374a in the high-grade glioma tissues were lower than those in the low-grade glioma tissues (P=0.027).

miR-374a is downregulated in glioma tissues. To validate the results that were obtained by the microarray analysis, the expression levels of miRNA-374a we evaluated in glioma (n=42) and normal brain tissues (n=5) by RT-qPCR. Significantly

lower miR-374a expression levels were observed in glioma tissues compared with those in the adjacent normal tissues and the expression of miR-374a decreased with increasing glioma grade (P<0.05; Fig. 1B and C). These RT-qPCR data confirmed that the results of the microarray analysis were reliable.

Association of miR-374a expression levels with clinico-pathological characteristics and OS of glioma patients. The association of the miR-374a expression levels with the demographic and clinicopathological characteristics of the glioma patients was then assessed (Table II). The median expression levels of miR-374a were used as a cut-off value, and all patients were divided into a high-level group (n=21) and a low-level group (n=21). The expression levels of miR-374a were associated with the KPS score (P=0.032) and the WHO grade (P=0.028; Table II). However, the miR-374a expression levels were not significantly associated with any of the other parameters assessed, including age and sex (P>0.05).

Kaplan-Meier curves and the log-rank test were used in order to evaluate the prognostic value of miR-374a in glioma. The results indicated that patients with low expression levels of miR-374a had a significantly shorter OS than those with high miR-374a expression levels (P=0.021; Fig. 1D). Furthermore, Cox regression analysis indicated that the expression levels of miR-374a were associated with the OS of glioma patients (hazard ratio, 0.472; 95% confidence interval, 0.125-1.733; P<0.05) and the WHO grade was also significantly associated with the OS of glioma patients (hazard ratio, 1.914; 95% confidence interval, 1.362-3.885; P<0.05; Table III) Collectively, these data suggest that miR-374a may be a prognostic factor regarding OS in patients with glioma.

Target prediction and functional analysis. To elucidate the potential biological function of miR-374a in glioma, the target genes of miR-374a were predicted using the TargetScan, miRTarBase, StarBase and miRDB online analysis tools. A total of 35 overlapping genes among the four different tools were identified (Fig. 2). Subsequently, a functional enrichment analysis was performed to investigate the biological functions of these consensus target genes. The enriched GO terms in the categories biological process (BP), cellular component and molecular function were identified. In the category BP, the target genes were mostly involved in metabolic processes (Fig. 3).

KEGG pathway analysis and PPI network. The KEGG pathways of the miR-374a target genes were predicted using DIANA-miRPath in order to identify miRNA-mRNA regulatory signaling pathways in glioma (21-23). The most significant biological pathways were associated with tumor progression from glial progenitor cells to primary GBM, including calcium metabolism, ErbB, mammalian target of rapamycin (mTOR) and several cell cycle-associated pathways (Fig. 4A). PPI network analysis of all target genes using the STRING database revealed 125 interactions involved. The nodes with a degree of interaction of ≥7 were defined as hub genes, including cyclin D (CCND)1, specificity protein 1 (Sp1), cyclin-dependent kinase (CDK)4 and CDK6 (Fig. 4B).

Table I. Differential expression miRNAs between high-grade and low-grade glioma groups.

miRNA ID	log2 fold change	P-value
hsa-let-7a-5p	-3.10	0.010
hsa-let-7b-5p	-2.28	0.032
hsa-let-7c-5p	-2.23	0.012
hsa-let-7d-5p	-2.82	0.018
hsa-let-7e-5p	-3.46	0.022
hsa-let-7f-5p	-3.29	0.011
hsa-miR-19a-3p	-2.60	0.008
hsa-miR-19b-3p	-3.02	0.021
hsa-miR-20a-5p	-2.31	0.017
hsa-miR-221-3p	2.58	0.026
hsa-miR-340-5p	-4.15	0.044
hsa-miR-374a-5p	-4.43	0.027
hsa-miR-886-3p	3.82	0.001
hsa-miR-9-3p	-3.79	0.049

miRNA/miR, microRNA; hsa, *Homo sapiens*.

Discussion

miRNAs are regulators of post-transcriptional gene expression and are involved in multiple complex pathways (24). A large number of studies have reported that certain miRNA are involved in the regulation of tumor progression, and may have tumor-promoting or tumor-suppressive roles with regard (25-27). miRNAs may be used as prognostic indicators and therapeutic targets in gliomas (28,29). In the present study, comprehensive miRNA profiling of high-grade vs. low-grade gliomas was performed by microarray analysis. A total of 15 significantly altered miRNAs in high-grade vs. low-grade gliomas were identified. In addition, a novel miRNA, miR-374a, was identified to be differentially expressed between normal and glioma tissues, which has not been previously reported. Therefore, miR-374a was selected for subsequent investigation. It was demonstrated that miR-374a was significantly lower expressed in high-grade vs. low-grade gliomas. The miRNA expression was verified by RT-qPCR in order to confirm the results of the microarray profiling. Furthermore, the present results indicated that miR-374a may be considered a potential prognostic biomarker in glioma due to the association of its expression levels with the long-term survival of glioma patients.

A number of studies have also reported that the abnormal expression of miR-374a are involved in multiple types of cancer. Li *et al* (30) revealed that deregulation of miR-374a may be involved in the development and regulation of cisplatin resistance in ovarian cancer cells. Wu *et al* (31) demonstrated that the expression levels of miR-374a were significantly lower in lung adenocarcinoma compared with those in the adjacent normal tissues. Furthermore, regulation of transforming growth factor α gene expression by miR-374a inhibited the proliferation, migration and invasion of lung adenocarcinoma cells (31). Slattery *et al* (32) demonstrated that the expression levels of miR-374a were downregulated in colorectal cancer,

Table II. Association of the expression level of miR-374a with clinicopathological factors of glioma.

Clinicopathological feature	Patients (n)	miR-374a expression (n)		P-value
		Low	High	
Age (years)				0.334
≥50	15	6	19	
<50	27	15	12	
Sex				0.533
Male	24	11	13	
Female	18	10	8	
WHO grade				0.028
II	13	5	8	
III/IV	29	16	13	
KPS score				0.032
≥90	11	2	9	
<90	31	9	12	

KPS, Karnofsky performance status; WHO, World Health Organization; miR, microRNA.

Table III. Univariate and multivariate analyses of prognostic factors in patients with glioma.

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥50 vs. <50 years)	1.414	0.591-3.384	0.436	1.031	0.421-2.524	0.946
Sex (male vs. female)	1.19	0.497-2.851	0.696	1.155	0.444-3.007	0.768
KPS (≥90 vs. <90)	0.274	0.089-0.844	0.024	1.025	0.390-2.699	0.056
WHO grade (II vs. III/IV)	1.51	1.163-2.98	0.027	1.914	1.362-3.885	0.036
miR-374a expression (low vs. high)	0.379	0.154-0.936	0.021	0.472	0.125-1.733	0.016

KPS, Karnofsky performance status; WHO, World Health Organization; miR, microRNA; HR, hazard ratio; CI, confidence interval.
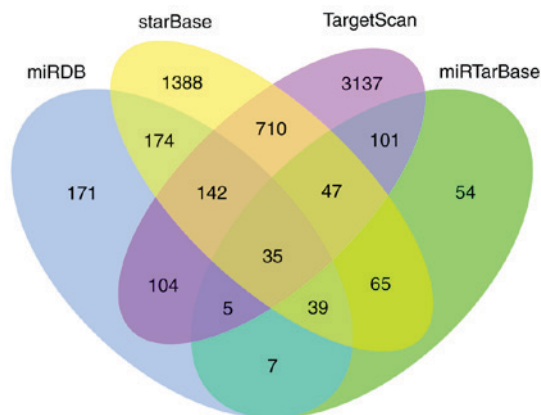

Figure 2. Venn diagram of overlapping target genes predicted using different online analysis tools (TargetScan, miRDB, starbase and miRTarBase).

whereas low miR-203 expression levels were associated with worse clinicopathological data and shorter OS time. However, Xu *et al* (33) reported that miR-374a acts as a tumor promoter in gastric cancer, where it and promotes cell proliferation, migration and invasion via the regulation of SRC kinase signaling inhibitor 1 expression levels. In addition, Pan *et al* (34) highlighted that the expression levels of miR-374 were decreased in glioma tissues and were associated with the prognosis of glioma patients, which is consistent with the results of the present study.

It has been reported that the aberrant expression of certain miRNAs is associated with the development of cancer via the abnormal regulation of multiple BPs and signaling pathways (35). To further elucidate the molecular function of miR-374a and its target genes, functional enrichment analyses of the target genes in GO terms and KEGG pathways were performed. The GO analysis demonstrated that the terms in the category BP included the regulation of the RNA transcription and the nucleic acid metabolic processes. Furthermore, several of the enriched pathways were associated with tumorigenesis, including ErbB, mTOR and cell cycle signaling pathways. The ErbB pathway is associated with tumor progression in the majority of cancer types, including glioma (36,37). Furthermore,
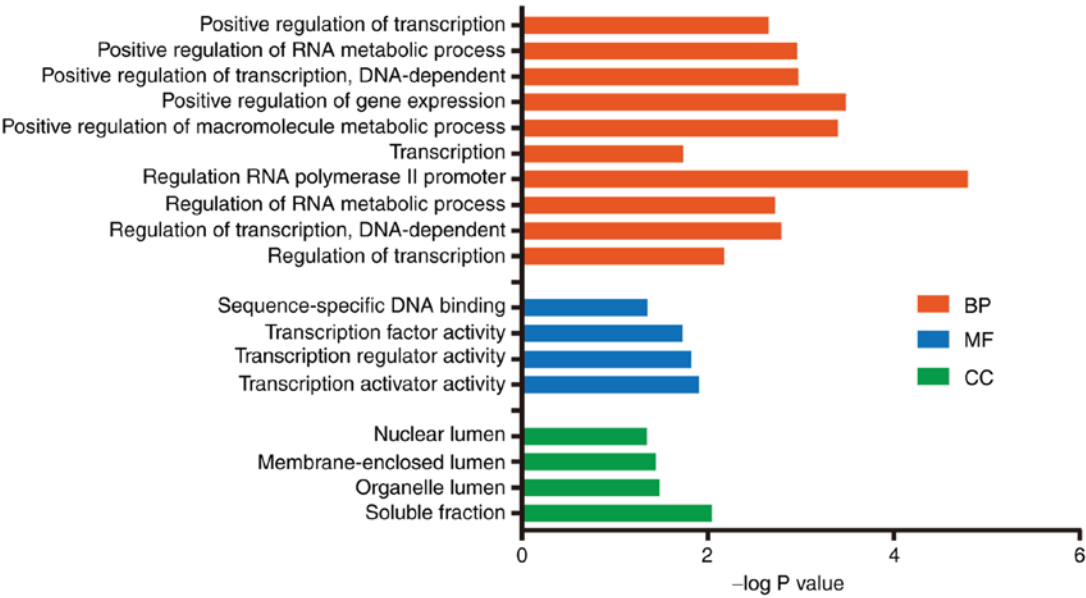
Figure 3. Significantly enriched Gene Ontology terms in the categories BP, MF and CC of the 35 consensus target genes. BP, biological process; MF, molecular function; CC, cellular component.
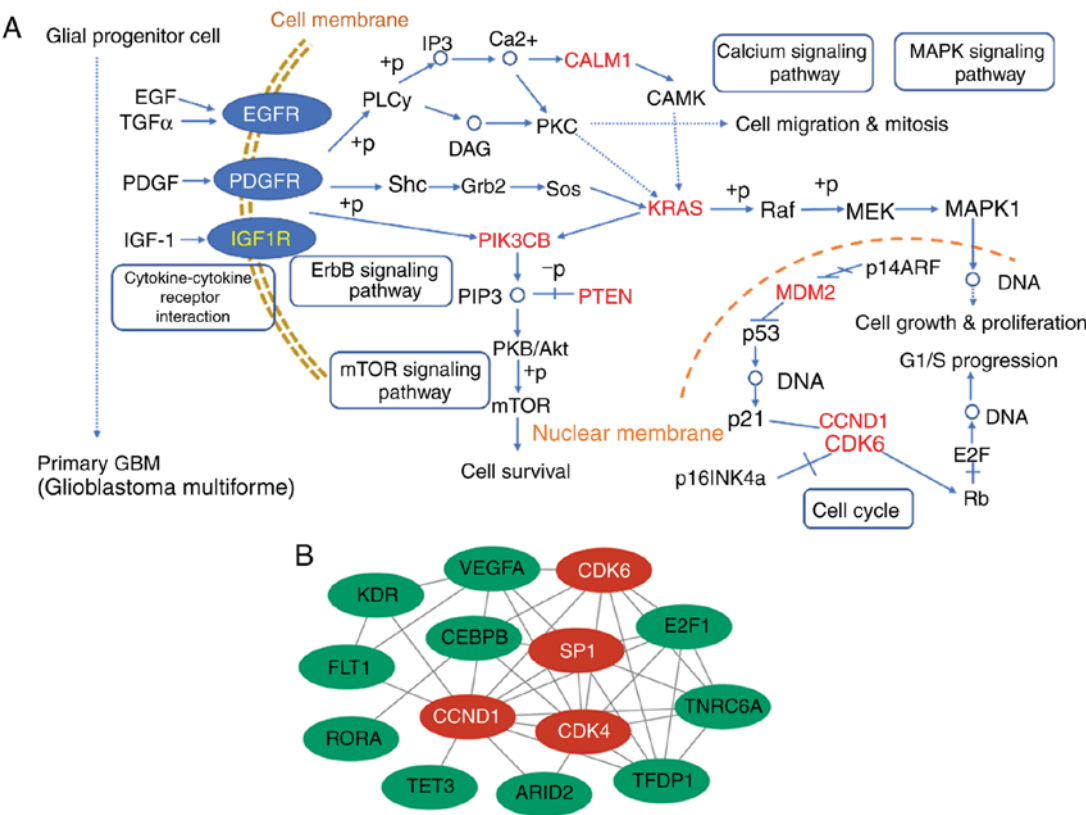

Figure 4. (A) KEGG pathway analysis based on predicted targets of miR-374a. These targets were identified in the Glioma Pathway using the DIANA-mirPath tool. The red color denotes the target gene of miR-374a. (B) Protein-protein interaction network constructed from target genes; red circles indicate hub genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA/miR, microRNA; GBM, glioblastoma multiforme.

it was reported that the mTOR pathway is a crucial signaling pathway involved in the development of glioma (38).

In addition, four hub genes, CCND1, SP1, CDK6 and CDK4, were identified from the PPI of predicted target genes of miR-374a, which may be directly or indirectly involved in the development of glioma. CCND1 is a protein required for the progression from G1 phase to the S phase of the cell cycle. CCND1 overexpression is associated with early cancer onset (39) and tumor progression and decreased Fas expression, leading to increased chemotherapeutic resistance and protection from apoptosis (40). CDK4 and CDK6 are two members of the CDK family that bind to CCND1. A dysregulation of

CDK4/6 may promote GBM proliferation; however, CDK4/6 kinase inhibitors were demonstrated to inhibit cell proliferation in subcutaneous glioma models (41). Sp1 belongs to the Sp/Kruppel-like factor family of transcription factors and is widely expressed in gliomas. Sp1 has an important role in the activation of oncogenes required for tumor survival (42). Guan et al (43) demonstrated that SP1 is upregulated in human glioma and may serve as a prognostic marker.

The primary limitation of the present study is that no luciferase reporter assays of the hub genes (CCND1, SP1, CDK6 and CDK4) were performed to validate the target genes of miR-374a. Therefore, further analyses are required to determine the mechanisms in the processes of malignant progression in gliomas. Future studies will use PCR to verify the expression levels of miR-374a in glioma cells and a combination of other molecular markers to successfully identify patients with OS.

In conclusion, the present study demonstrated that the downregulation of miR-374a is associated with reduced survival in glioma patients. miR-374a may be a potential prognostic factor for patients with glioma. However, the results of the present study should be verified using a larger sample size and further experimental research is required to confirm the functions of miR-374a in the progression of glioma.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YP and QD initiated the project. QD, ML, GY, MW and JH performed the experiments. QD, ML, QX and GY analyzed the data. QX, JH, SL and XM generated the figures. YP, QD, GY and ML wrote the manuscript. All co-authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Second Hospital of the Lanzhou University. Written informed consent was provided by each patient or their guardians prior his/her participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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