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Potentials of miR-9-5p in promoting epileptic seizure and improving survival of glioma patients

Shenglin Wang[1,2], Xuzhi He[3], Nana Bao[1,2], Mingyue Chen[1,2], Xiaomi Ding[1,2], Ming Zhang[1,2], Li Zhao[1,2], Shunxian Wang[1,2*] and Guohui Jiang[1,2*]

Abstract

Background: Epilepsy affects over 70 million people worldwide; however, the underlying mechanisms remain unclear. MicroRNAs (miRNAs) have essential functions in epilepsy. miRNA-9, a brain-specific/enriched miRNA, plays a role in various nervous system diseases and tumors, but whether miRNA-9 is involved in epilepsy and glioma-associated epilepsy remains unknown. Therefore, we aimed to explore the potential role of miR-9-5p in seizures and its effect on the survival of glioma patients, in order to provide new targets for the treatment of epilepsy and glioma.

Methods: The YM500v2 database was used to validate the expression of hsa-miR-9-5p in tissues. Moreover, qRT-PCR was performed to investigate the expression of miR-9-5p in temporal lobe epilepsy patients and rats with lithium-pilocarpine-induced seizures. Recombinant adeno-associated virus containing miR-9-5p was constructed to overexpress miR-9-5p in vivo. The effects of miR-9-5p on the behavior and electroencephalographic activities of the lithium-pilocarpine rat model of epilepsy were tested. Bioinformatics analysis was used to predict the targets of miR-9-5p and explore its potential role in epilepsy and glioma-associated epilepsy.

Results: The expression of miR-9-5p increased at 6 h and 7 days after lithium-pilocarpine-induced seizures in rats. Overexpression of miR-9-5p significantly shortened the latency of seizures and increased seizure intensity at 10 min and 20 min after administration of pilocarpine ($P < 0.05$). Predicted targets of miR-9-5p were abundant and enriched in the brain, and affected various pathways related to epilepsy and tumor. Survival analysis revealed that overexpression of miR-9-5p significantly improved the survival of patients from with low-grade gliomas and glioblastomas. The involvement of miR-9-5p in the glioma-associated epileptic seizures and the improvement of glioma survival may be related to multiple pathways, including the Rho GTPases and hub genes included SH3PXD2B, ARF6, and ANK2.

Conclusions: miR-9-5p may play a key role in promoting epileptic seizures and improving glioma survival, probably through multiple pathways, including GTPases of the Rho family and hub genes including SH3PXD2B, ARF6 and ANK2. Understanding the roles of miR-9-5p in epilepsy and glioma and the underlying mechanisms may provide a theoretical basis for the diagnosis and treatment of patients with epilepsy and glioma.

Keywords: Epilepsy, Seizure, Glioma, miR-9-5p, Survival

Background

Epilepsy is one of the most commonly occurring and devastating chronic neurologic disorders, which manifests as unprovoked, recurrent seizures [1, 2]. It affects over 70 million people worldwide, ranging from

*Correspondence: 108846217@qq.com; neurodoctor@163.com

[1] Department of Neurology, Affiliated Hospital of North Sichuan Medical College, No.1 Maoyuan South Road, Nanchong 637000, China
Full list of author information is available at the end of the article

neonates to the elderly [1, 3]. Social isolation, internal stigma, and stress from unpredictable seizures represent significant barriers to normal life for patients and their families [4, 5]. Unfortunately, the morbidity and mortality rates of epilepsy remain unchanged, and up to one-third of these patients still suffer from medically refractory seizures, despite numerous therapeutic progresses [6–8]. This is mainly because that the current treatments primarily suppress seizures rather than correcting the mechanisms underlying the pathogenesis of epilepsy [8]. Further, these underlying molecular mechanisms are not fully understood.

Both epilepsy and glioma are localized, highly energy-consuming diseases of the brain. Large-scale dynamic changes in gene expression are considered to be the basis for the causal pathogenic processes of epilepsy, such as ion channel regulation, glial proliferation, neuronal death, inflammation, modulation of neurotransmitter receptors, synaptic remodeling, cell proliferation and differentiation, and migration [9–14]. MicroRNAs (miRNAs) represent a diverse class of small (~ 22 nucleotides), non-coding RNAs that negatively modulate gene expression by binding to complementary sequences of the 3′ untranslated region in most cases [15]. Emerging evidence shows that, as critical regulators of brain development and function, various miRNAs play important roles in neurological diseases, including epilepsy [10, 16–20].

miRNA-9 (miR-9), a brain-specific/enriched miRNA, can regulate a variety of signaling pathways at the post-transcriptional level, mainly related to neurogenesis, and is the core of the gene network controlling the progenitor state [15, 21]. Intriguingly, this miRNA may exert opposite effects in different nervous system diseases. miR-9 plays a protective role during pathological processes of stroke [22–26], Alzheimer's disease [27, 28], spinal cord injury [29], and multiple sclerosis [30], while it aggravates neurotoxicity in *N*-methyl-4-phenylpyridinium iodide-induced Parkinson's disease by targeting Sirtuin 1 [21, 31]. In particular, the effect of miR-9 on glioma remains controversial. While some studies have reported its role in the development and progression of glioma and association with an unfavorable prognosis in human gliomas [21, 32], other studies have shown that miR-9 can inhibit glioma growth [33–37]. Previous studies have demonstrated that miR-9 is significantly upregulated in the epileptic tissues from humans and animal models [38–41]. However, it remains largely unknown if or how miR-9 affects the primary epilepsy and secondary glioma-associated epilepsy.

Therefore, in this study, we set out to evaluate the contributions of miR-9-5p to epilepsy.

Materials and methods

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

miR-9-5p-specific primer (5′-UCUUUGGUUAUCUAGCUGUAUGA-3′) was designed and synthesized by Guangzhou Ribo BioCompany (Guangzhou, China) [42]. Bulge-Loop™ qRT-PCR was performed [43, 44] in two steps: (1) the reverse transcription reaction using miR-9- and U6-specific stem-loop reverse transcription primers, and (2) fluorescent quantitative PCR using SYBR Green fluorescent dye and specific forward/reverse primers.

Construction of recombinant adeno-associated virus (rAAV) carrying miR-9-5p

To overexpress miR-9-5p in vivo, rAAV containing miR-9-5p was constructed by inserting the precursor miR-9-5p gene fragment into the AAV plasmid. The recombinant expression plasmid was then cotransfected into AAV-293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV replication and capsid genes). Next, the AAV virus particles were collected from the infected AAV-293 cells. Finally, the virus carrying miR-9-5p was condensed and purified for animal experiments. PCR verified the expression of miR-9-5p in rAAV and the concentration of the virus was 1.41E+13 v.g./ml.

Animals

All procedures involving animals were conducted in strict compliance with the Chinese Animal Welfare Act, and approved by the Animal Experimentation Ethics Committee of the North Sichuan Medical College (approval number NSMC(A) 2021 (21)). Male Sprague-Dawley rats weighing 180–220 g (6–8 weeks old) were purchased from the Animal Experiment Center of North Sichuan Medical College. Animals were housed at 22–24 °C with 50–60% humidity under a 12-hour light-dark cycle (lights on at 8:00 am), with free access to food and water.

Experimental animal grouping

The rats were anesthetized with intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg) and eye lubrication was used to minimize drying throughout the procedure. One microliter of AAV was stereotactically injected into each of the bilateral hippocampi (0.1 µl/min; coordinates: AP, −3.6 mm, ML, −2.8 mm, DV, −3.5 mm) using a microinjector (Gaoge, Shanghai, China) (10 µl capacity) within 10 min [45]. Rats in the experimental group were infected with the miR-9-5p-carrying virus ($n=11$), while those in the vehicle group were injected with an empty adenovirus ($n=11$). To permit spreading of the virus and minimize reflux when the needle was retracted, the needle was retained in place for 10 min post-injection, then it was slowly withdrawn. After surgery, a heating pad was

used to maintain the rat's temperature at 37 °C for recovery. The entire process lasted 2 to 4 h.

Animal model of epilepsy

Three weeks after viral infection, lithium chloride and pilocarpine was used to induce status epilepticus (SE), which reproduces most of the features of human temporal lobe epilepsy (TLE). Rats receiving either vehicle or miR-9-5p-carrying virus were treated with intraperitoneal injections of lithium chloride (127 mg/kg) and atropine (1 mg/kg), 20 h and 30 min before the first administration of pilocarpine by intraperitoneal injection (35 mg/kg), respectively. Seizures were scored according to the Racine's standard criteria [46]. If no seizure of grade 4 or higher occurred within 30 min of the first dose, the dose of pilocarpine was increased by 20% every 10 min until the occurrence of level-4–5 seizures. However, the number of pilocarpine injections per animal did not exceed five [47]. Sixty minutes after SE initiation, the rats were administered with intraperitoneal diazepam (10 mg/kg) to terminate the continuous seizures [48]. Only animals with seizures of level 4–5 were evaluated.

Surgical procedures and electrophysiological recordings

The rats were anesthetized with 1% sodium pentobarbital (40 mg/kg, intraperitoneal) and fixed on a stereotaxic apparatus (RWD Life Science Co., Ltd., China). The subcutaneous tissue and periosteum were separated bluntly with cotton swabs to minimize the extent of injury and bleeding in order to fully expose the skull. The position of the right hippocampus (AP − 3.6 mm, ML − 2.8 mm, DV − 3.5 mm) was determined and marked on the skull surface [45]. The skull window was covered with dental cement H-frame for recording hippocampal local field potentials (LFPs) [49, 50].

To record neural activity, the rats were implanted with recording electrodes (4 × 4 platinum-iridium alloy electrode array, each 25 μm in diameter). The lower end of the microfilament electrode was placed close to the brain tissue using a microdriver [27]. LFPs were pre-amplified (× 1000), filtered (0.1–1000 Hz), and digitized at 4 kHz using an OmniPlex® D Neural Data Acquisition System (Plexon Inc., Dallas, TX) [45, 51]. Baseline LFPs were recorded for 10 min followed by intraperitoneal injection of atropine. After a 30-min interval, pilocarpine was administered to induce seizures in both groups, and recordings were made for a total of 120 min after onset of seizures of level 4–5.

Human participants

Temporal lobe cortical tissueswere randomly chosen from 220 specimens in the epileptic brain tissue bank from Chongqing Medical University. A total of 24 patients with medically refractory TLE, including 13 males and 11 females, were analyzed [50]. The mean age of the patients was 29.79 ± 1.71 years (range: 14–47) and the mean course of the disease was 10.29 ± 0.98 years (range: 3–19). At least three antiepileptic drugs were demonstrated to be ineffective in these patients. The control group included 12 age- and sex-matched patients with traumatic brain injury or hematoma clearance who were treated with temporal cortical excision (with no history of epilepsy or epileptic drug exposure). Informed consent was obtained from all patients or their relatives for the use of brain tissues in the experimental procedures. The research protocol was conducted in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and the requirements of the ethics committee of Chongqing Medical University.

Visualization of the expression profile of miR-9-5p in human tissues

The expression profile of miR-9-5p in human tissues was detected and visualized using the database YM500v2 (http://ngs.ym.edu.tw/ym500v2/index.php), which incorporating 8,105 smRNA-seq datasets from TCGA involved in those of primary tumors, paired normal tissues, peripheral blood mononuclear cell (PBMC), recurrent tumors and metastatic tumors [52].

Target gene prediction

Target genes of hsa-miR-9-5p were predicted using TargetScan (https://www.targetscan.org/vert_80/), miRDB (http://mirdb.org/), and miRwalk (http://mirwalk.umm.uni-heidelberg.de/) softwares. Venn diagrams were generated with hiplot (https://hiplot.com.cn/basic/dendrogram) [53, 54].

Gene chip data acquisition

To confirm the role of hsa-miR-9-5p in tumor-induced epilepsy, the gene expression profile GSE32534 was obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Five formalin-fixed paraffin-embedded peritumoral cortical tissue sections were obtained from low-grade glioma patients, divided into seizure-paired and non-seizure groups (Table 1). GSE32534 was quantified using the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

Table 1 Sample information of the gene expression profile dataset GSE32534

Accession	Title	Source name	Gender	Tissue	Disease state	Tumor type
GSM805925	Epilepsy, sample 1	FFPE peritumoral sections	Male	peritumoral cortex	Epilepsy	astrocytoma
GSM805926	Epilepsy, sample 2	FFPE peritumoral sections	Male	peritumoral cortex	Epilepsy	ganglioglioma
GSM805927	Epilepsy, sample 3	FFPE peritumoral sections	Female	peritumoral cortex	Epilepsy	oligodendroglioma
GSM805928	Epilepsy, sample 4	FFPE peritumoral sections	Male	peritumoral cortex	Epilepsy	ganglioglioma
GSM805929	Epilepsy, sample 5	FFPE peritumoral sections	Male	peritumoral cortex	Epilepsy	astrocytoma
GSM805930	No Epilepsy, sample 1	FFPE peritumoral sections	Female	peritumoral cortex	No epilepsy	ganglioglioma
GSM805931	No Epilepsy, sample 2	FFPE peritumoral sections	Male	peritumoral cortex	No epilepsy	ganglioglioma
GSM805932	No Epilepsy, sample 3	FFPE peritumoral sections	Female	peritumoral cortex	No epilepsy	oligodendroglioma
GSM805933	No Epilepsy, sample 4	FFPE peritumoral sections	Male	peritumoral cortex	No epilepsy	ganglioglioma
GSM805934	No Epilepsy, sample 5	FFPE peritumoral sections	Male	peritumoral cortex	No epilepsy	astrocytoma

FFPE formalin-fixed paraffin-embedded

Functional and tissue enrichment analysis

For functional enrichment analyses, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the TCGA biolinks package [55]. Tissue enrichment analysis was conducted using DAVID v6.8 (https://david.ncifcrf.gov/summary.jsp) and plotted using hiplot [53, 54].

Recognition and analysis of differentially expressed genes (DEGs)

DEGs were analyzed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), which was included in the GEO database. GSE32534 data were divided into the non-epileptic glioma group or epileptic glioma group [56]. Genes with the absolute value of the logarithm (base 2) fold change (logFC)>0.3 and $P<0.05$ were considered as DEGs. The volcano plot and heatmap of the DEGs were plotted at http://www.bioinformatics.com.cn.

Survival analysis

Survival analysis was performed with the online Oncolnc survival analysis server (http://www.oncolnc.org, data gathered from TCGA). Tumor tissue versus normal tissue from the TCGA database was used.

Protein-protein interaction (PPI) network construction and module analysis

PPI data of predicted targets of hsa-miR-9-5p involved in the glioma-associated epilepsy were obtained from the STRING database (version 11.0, https://string-db.org/), which collects and integrates known and predicted PPI data. The results were then imported into the Cytoscape software and visualized. The top 10 hub genes and significant modules in the PPI network were identified using the MCODE plugin and the MCC method of cytoHubba plugin of the Cytoscape software.

Statistical analysis

All data were analyzed using the GraphPad Prism 8.0.2 software (GraphPad Software, Inc., La Jolla, CA) or the SPSS 25.0 software (IBM, Inc., CA). Values are expressed as the mean ± standard deviation. Student's *t*-test and Wilcoxon ranked-sum test were used for comparison of normally distributed continuous variables and categorical variables, respectively. The Fisher's exact test was used to compare rates. $P<0.05$ was considered as significantly different. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns: no statistically significant difference.

Results

miR-9-5p levels increase in rat hippocampus at 6 h and 7 days after SE

miR-9 has two mature forms, miR-9-5p and miR-9-3p [21, 57], which are often called miR-9 and miR-9*, respectively, because of the preferential use of the 5′ strand in deuterostomes [21]. Using the YM500v2 database, both forms of miR-9 were found to be enriched in the human brain, especially hsa-miR-9-5p [52] (Fig. 1a, b). To address the role of miR-9-5p in epileptic seizures, we investigated its expression levels in TLE patients and rats with lithium-pilocarpine-induced seizures using qRT-PCR. The miR-9-5p expression in temporal cortical tissues of TLE patients did not significantly differ from that in controls ($P>0.05$) (Fig. 1c). Interestingly, qRT-PCR results showed that the miR-9-5p expression in the hippocampus of lithium-pilocarpine-treated rats was significantly higher at 6 h and 7 days after SE than that in the vehicle group (Fig. 1d), but not at 24 h, 21 days, or 60 days

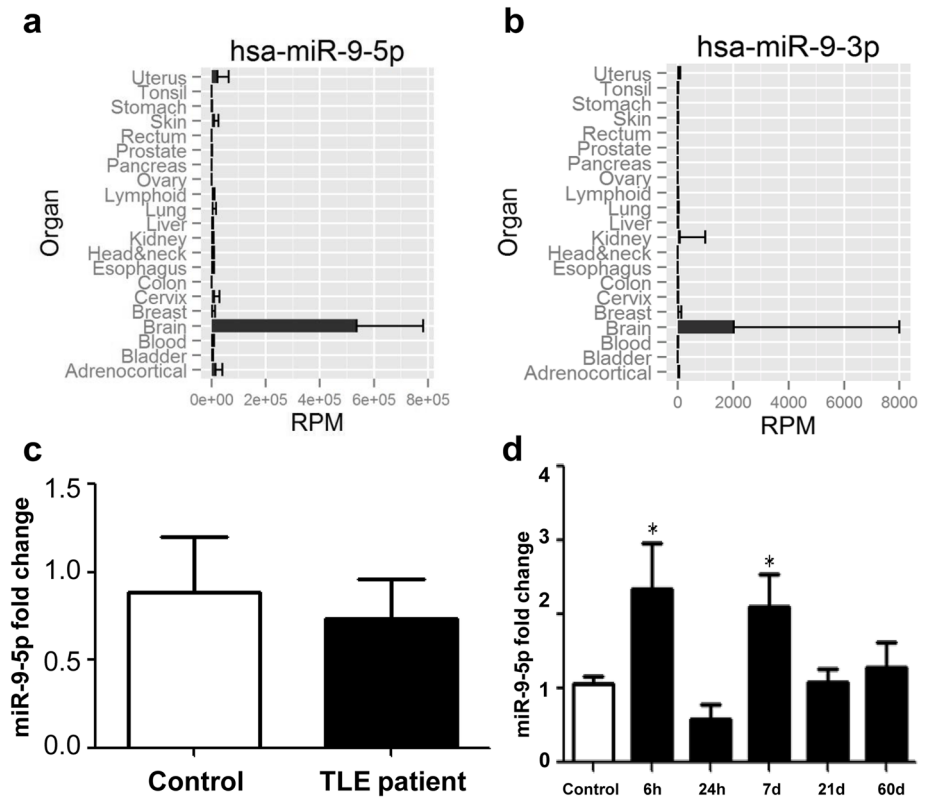

Fig. 1 Expression of miR-9-5p in epileptic and non-epileptic tissues. **a** Expression of miR-9-5p in human tissue; **b** Expression of miR-9-3p in human tissue; **c** Expression level of miR-9-5p in the temporal lobe of TLE patients and controls; **d** Expression of miR-9-5p in the hippocampal tissues of rats was significantly increased at 6 h and 7 days after SE, compared to the control group (*$P < 0.05$). RPM, reads per million mapped reads

after SE ($P > 0.05$). These data indicate that miR-9-5p may play a vital and complex role in epileptic seizures.

Overexpression of miR-9-5p significantly exacerbates the lithium-pilocarpine-induced epileptic seizures

Rats were randomly divided into the vehicle or miR-9-5p group ($n = 11$ in each group) to investigate the effects of miR-9-5p on lithium-pilocarpine-induced epileptic seizures. Seizure latency was defined as the duration from pilocarpine administration to the occurrence of first seizure of grade ≥ 4. Results showed that the latency of rats in the miR-9-5p group was significantly shorter than that of the vehicle group (Student's *t*-test, $P < 0.05$) (Fig. 2a). Moreover, the miR-9-5p group had significantly higher Racine scores than those in the vehicle group at 10 min and 20 min after seizure onset (grade 4 or higher) (Wilcoxon rank-sum test, $P < 0.05$) (Fig. 2b). The rate of seizures above grade 4 in the miR-9-5p group was 100% and that in the vehicle group was 81.8% (Fisher's exact test, $P > 0.05$). EEG recordings further revealed shorter latency and higher severity of epileptic seizures (Fig. 2d).

miR-9-5p target gene prediction and enrichment analysis

As mentioned above, miRNAs generally exert biological functions by suppressing the expression of specific target genes. Hence, we sought to predict the potential target genes of miR-9-5p using TargetScan, miRDB, and miRWalk softwares (Fig. 3a). Tissue enrichment analysis indicated that the predicted target genes of miR-9-5p were highly expressed in the human brain, especially in amygdala (Fig. 3b). GO and KEGG terms with corrected $P < 0.05$ were considered significantly enriched (Fig. 4). GO analysis consists of biological processes (BP), cell composition (CC), and molecular function (MF). BP analysis showed that the targets were mainly enriched in processes of extracellular matrix organization ($n = 6$), neurological system process ($n = 5$), and metencephalon development ($n = 3$). CC analysis showed that the targets were mainly enriched in the trans-Golgi network ($n = 6$), proteinaceous extracellular matrix ($n = 11$), and cell projection ($n = 14$). MF analysis showed that the targets were mainly enriched in actin binding ($n = 16$), phosphatidylinositol transporter activity ($n = 2$), and zinc ion

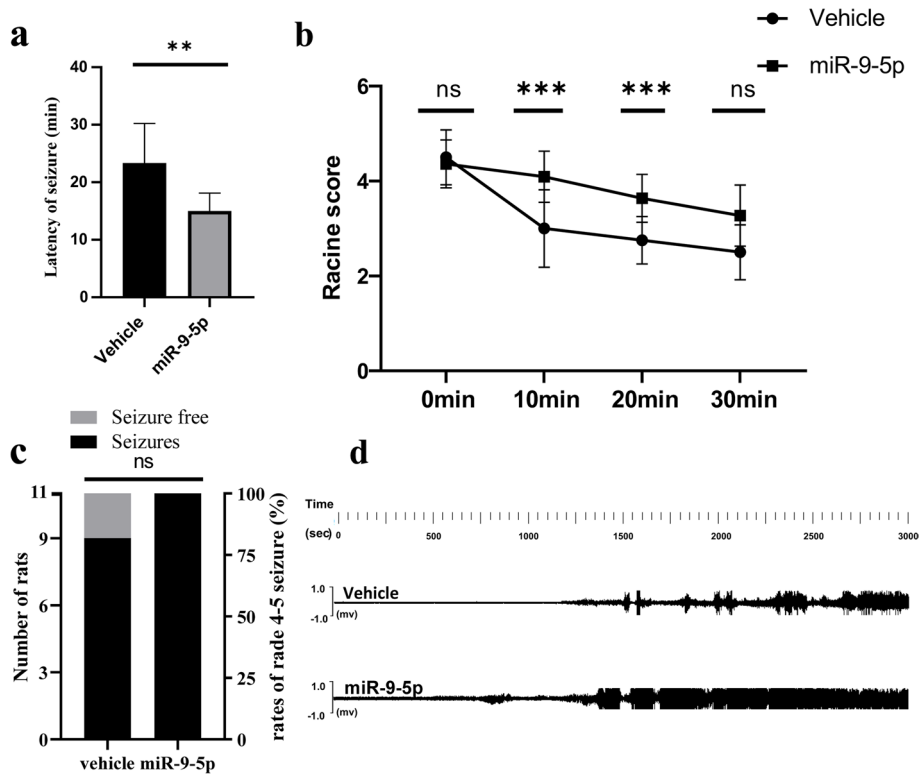

Fig. 2 Effects of miR-9-5p overexpression on lithium-pilocarpine-induced seizure activity in rats. **a** Effects of miR-9-5p overexpression on seizure latency. **b** Racine scores at different time points after seizure onset. **c** Comparison of seizure rate. **d** Representative EEG recordings of lithium-pilocarpine-induced seizures in the miR-9-5p and vehicle groups

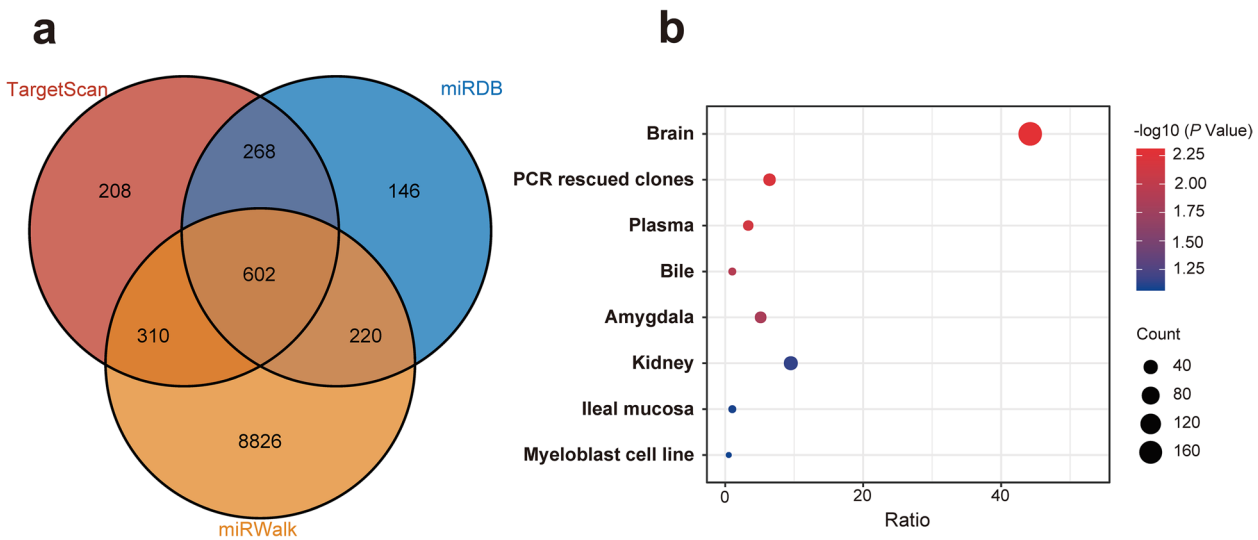

Fig. 3 Predicted target genes of hsa-miR-9-5p. **a** A total of 602 target genes of hsa-miR-9-5p were predicted by all of the Targetscan, miRDB, and miRwalk softwares. **b** Tissue enrichment analysis suggested that the target genes were most highly expressed in the brain, especially in the amygdala

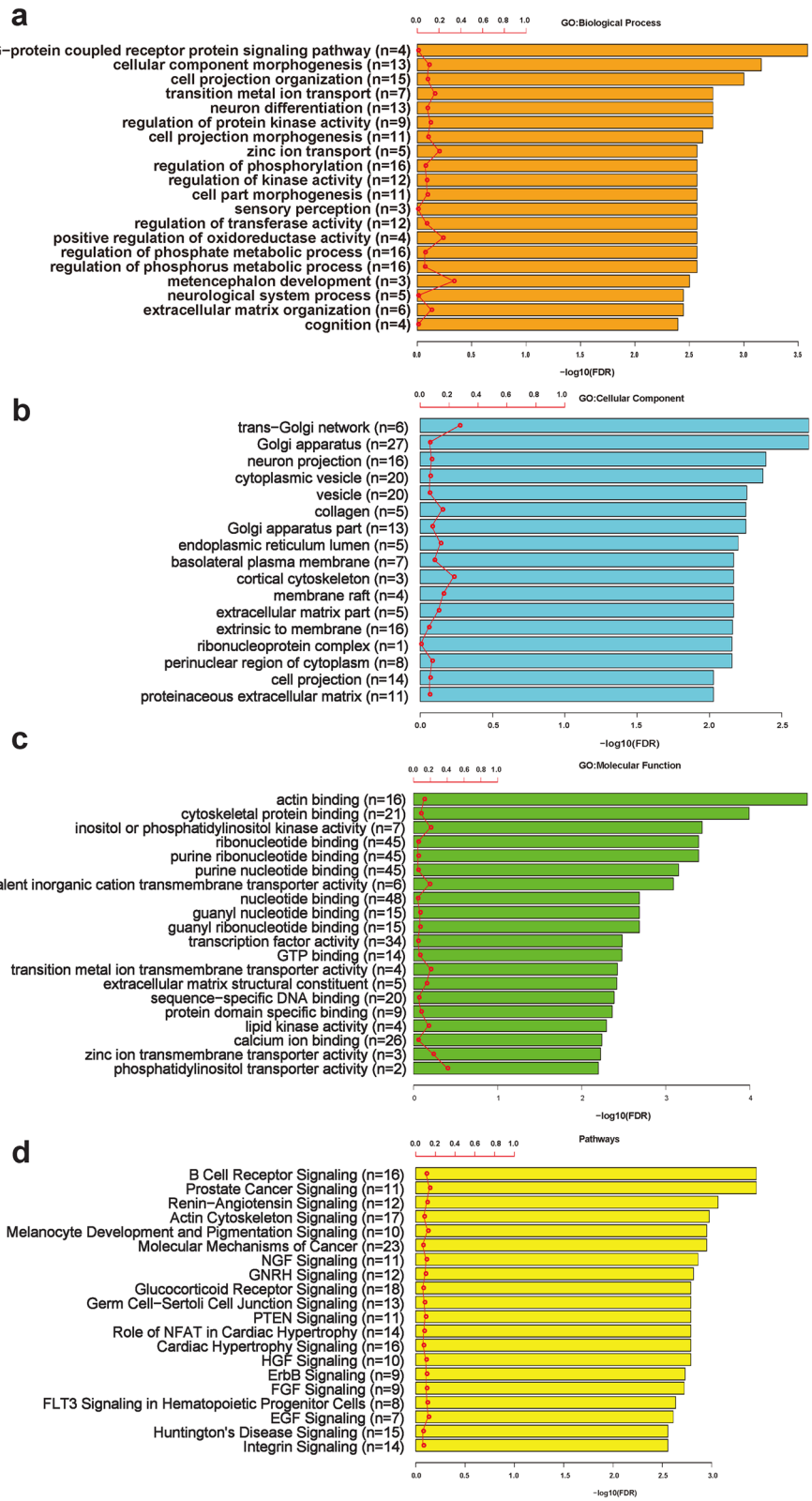

Fig. 4 Enrichment analysis of target genes of hsa-miR-9-5p. **a** GO (BP) enrichment analysis. **b** GO (CC) enrichment analysis. **c** GO (MF) enrichment analysis. **d** KEGG enrichment analysis

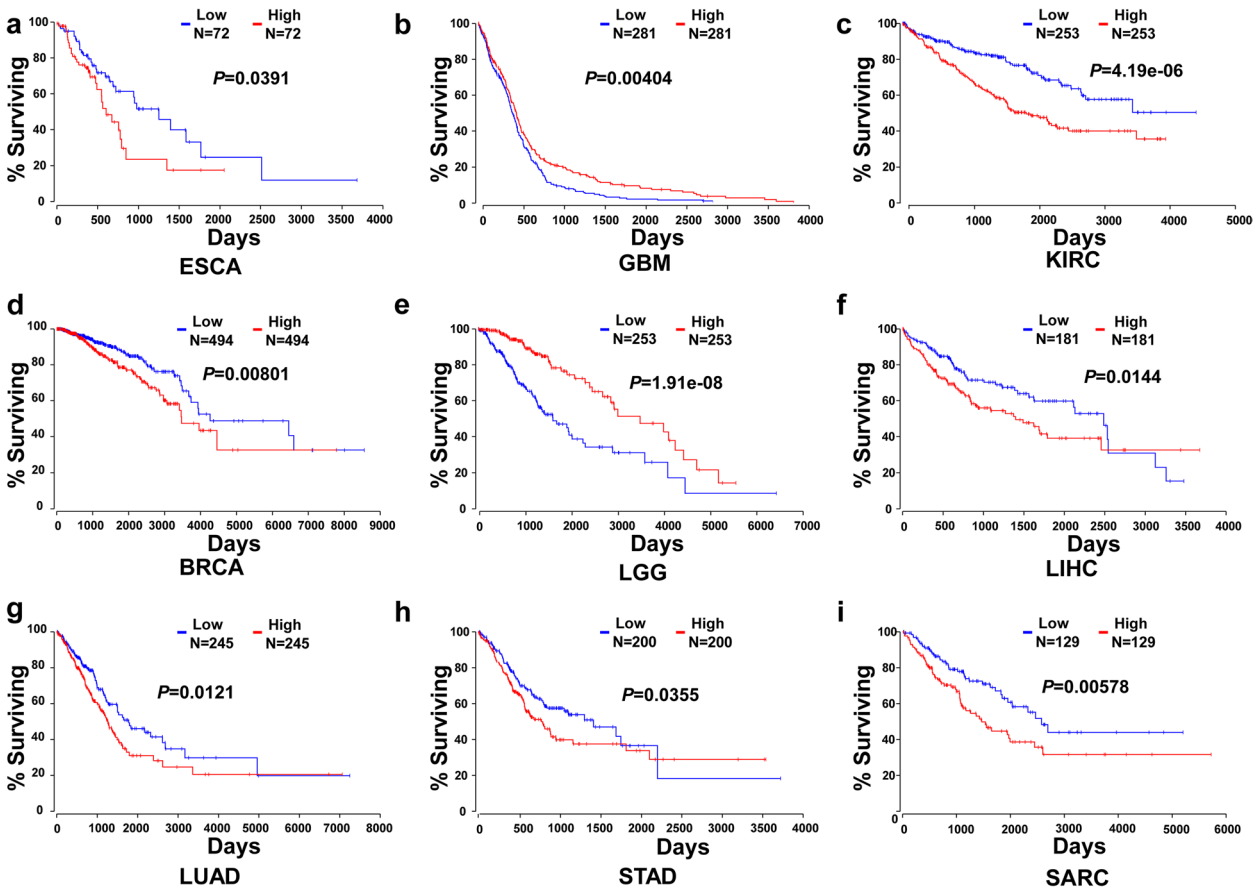

Fig. 5 Survival analysis of nine types of tumor

transmembrane transporter activity ($n=3$). In addition, a large number of terms were enriched in KEGG, especially those associated with cancer and epilepsy, such as molecular mechanisms of cancer ($n=23$), phosphatase and tensin homolog (PTEN) signaling ($n=11$) [58], prostate cancer signaling ($n=11$), fibroblast growth factor (FGF) signaling ($n=9$) [59, 60], and ErbB signaling ($n=9$) [61].

Hsa-miR-9-5p may play a critical role in human cancer, especially glioma

Enrichment analysis indicated that the hsa-miR-9-5p may regulate tumor-associated pathways. Thus, to investigate the potentially critical role of miR-9-5p in human cancer, Kaplan–Meier survival analysis involving 21 types of tumor was performed using OncoLnc [62]. The results showed that hsa-miR-9-5p might influence the survival from nine types of tumor ($P<0.05$). Increased hsa-miR-9-5p expression was correlated with poor survival from seven types of tumor including esophageal carcinoma (ESCA), kidney renal clear cell carcinoma (KIRC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), sarcoma (SARC), stomach adenocarcinoma (STAD), and breast invasive carcinoma (BRCA), while predicting longer survival from glioblastoma (GBM) and lower-grade glioma (LGG) (Fig. 5).

Hsa-miR-9-5p may play a critical role in glioma-induced epilepsy

Based on the results that high expression of hsa-miR-9-5p predicted longer survival in GBM and LGG, and with previous evidence indicating that the glioma-induced epilepsy may be associated with favorable prognosis [63–65], we hypothesized that hsa-miR-9-5p might play a key role in glioma-induced epilepsy. The gene expression profile dataset GSE32534 was used to identify DEGs between epilepsy and non-epilepsy patients with low-grade brain tumor, which were considered as risk genes (Fig. 6a, b) [62]. A Venn diagram was made to compare the DEGs (risk genes) with the miR-9-5p targets, which showed that 12.6% of the predicted targets were risk genes (Fig. 6c). Subsequently, for these targets of hsa-miR-9-5p associated with glioma-associated epilepsy, the PPI network and significant functional modules identified by MCODE plugin of the Cytoscape software were

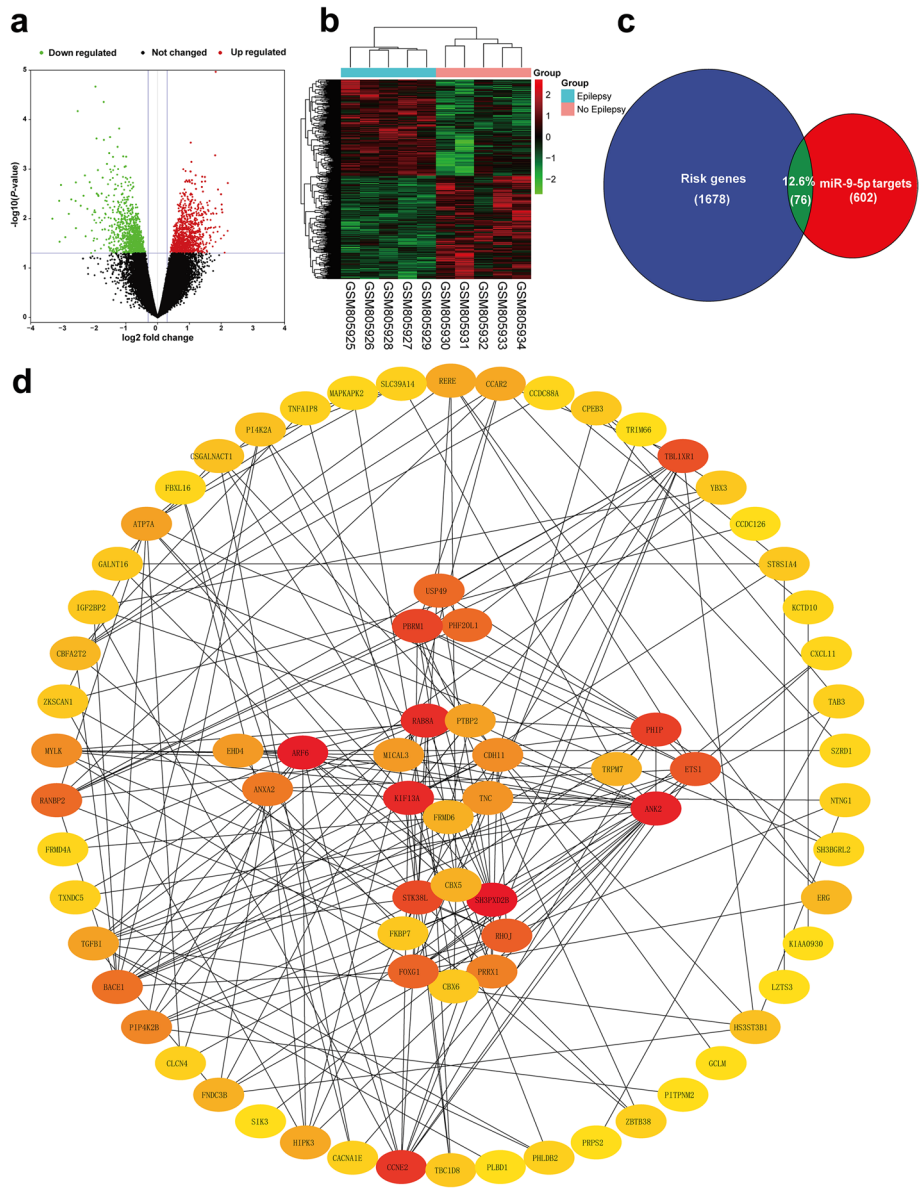

Fig. 6 Targets of hsa-miR-9-5p are associated with glioma-associated epilepsy. **a** Volcano plot of DEGs. **b** Heatmap of the DEGs. **c** Venn diagrams of overlap between DEGs (risk genes) and miR-9-5p targets. **d** Schematic diagram of PPI of targets of hsa-miR-9-5p associated with glioma-associated epilepsy. Five significant functional modules identified by MCODE were circled by other targets. For both nodes, a darker color indicates a higher MCC score

visualized (Fig. 6d). The top 10 hub genes were identified by the MCC method of cytoHubba plugin, including SH3 and PX domains 2A (*SH3PXD2B*), ADP ribosylation factor 6 (*ARF6*), ankyrin 2, neuronal (*ANK2*), kinesin family member 13A (*KIF13A*), member RAS oncogene family (*RAB8A*), cyclin E2 (*CCNE2*), pleckstrin homology domain interacting protein (*PHIP*), polybromo 1 (*PBRM1*), serine/threonine kinase 38 like (*STK38L*), and transducin (beta)-like 1 X-linked receptor 1 (*TBL1XR1*) (Table 2). GO analysis showed that these targets were mainly enriched in di- and trivalent inorganic cation transport ($n = 3$), cell projection organization ($n = 4$), and trans-Golgi network ($n = 2$) (Fig. 7). Only four pathways were enriched in KEGG analysis, which were regulation of actin-based motility by Rho ($n = 3$), chondroitin sulfate biosynthesis (late stages) ($n = 2$), signaling by Rho family GTPases ($n = 4$), and D-myo-inositol (1,4,5)-trisphosphate biosynthesis ($n = 2$).

Table 2 Basic information of top 10 hub genes

Rank	Gene	Full name	LogFC	Score
1	SH3PXD2B	SH3 and PX domains 2A	−0.50491	126
2	ARF6	ADP ribosylation factor 6	−0.18908	118
3	ANK2	ankyrin 2, neuronal	0.68974	113
4	KIF13A	kinesin family member 13A	0.477741	68
5	RAB8A	member RAS oncogene family	0.689748	64
6	CCNE2	cyclin E2	0.50645	62
7	PHIP	pleckstrin homology domain interacting protein	−0.10544	49
8	PBRM1	polybromo 1	−0.15817	48
9	STK38L	serine/threonine kinase 38 like	−0.52338	39
10	TBL1XR1	transducin (beta)-like 1 X-linked receptor 1	0.42959	37

Discussion

In this study, we confirmed that the expression of miR-9-5p increased at 6 h and 7 days after SE in hippocampal tissues from epileptic rat models. miR-9-5p overexpression aggravated SE, and the mechanism underlying this effect likely involved in various targets. Survival analysis indicated that miR-9-5p may lead to better glioma survival. These results revealed a previously unrecognized role of miR-9-5p in modifying epilepsy and in improving glioma survival; therefore, it may be a potential novel target of diagnosis and treatment for epilepsy and glioma.

Previous studies have reported different alterations of miR-9 expression in epilepsy [38–41]. For example, Kan et al. performed array-based genome-wide miRNA expression profiling, showing that miR-9 was significantly upregulated in hippocampal tissues from patients with mesial TLE [39]. In contrast, Risbud et al. performed microRNA array analysis on the whole hippocampus of lithium-pilocarpine-induced C57 mice, showing that miR-9 expression decreased at 4 h, 48 h, and 3 weeks following SE ($P < 0.05$) [40]. However, most previous studies did not distinguish miR-9-5p from miR-9-3p. Hence, we employed qRT-PCR and confirmed that miR-9-5p expression in the hippocampus of lithium-pilocarpine-treated rats was increased at 6 h and 7 days after SE, but not changed at 24 h, 21 days, or 60 days in rats. Similarly, we did not find altered expression of miR-9-5p in cortical tissues from patients with TLE. This indicates that the change of miR-9-5p expression is not consistent throughout the epileptic process. The dynamic change of miR-9-5p at various time points after SE may be associated with epilepsy. These findings reveal complex changes of miR-9-5p expression in epileptic tissues, which may be related to the complex regulation of miR-9-5p involved in epileptic seizures.

Previous studies have shown that miRNAs serve as key regulators of the pathophysiology of epilepsy [19, 66–70]. Our behavioral analysis showed that miR-9-5p overexpression reduced seizure latency, and significantly increased seizure grade at 10 min and 20 min after first seizure of grade >4, suggesting that this miRNA is pro-epileptic. miRNAs are involved in various biological functions via regulation of their target genes [71], so we next predicted the target genes of hsa-miR-9-5p by bioinformatics tools and performed tissue enrichment analysis and functional enrichment analysis [62]. We found that most of these targets were enriched in the brain, especially in the amygdala, which has also been previously suggested to be one of the key structures involved in epilepsy [72–75]. GO analysis indicated that these targets may be involved in neurological system processes associated with epilepsy, such as neuron differentiation [76], transition metal ion transport [77], G-protein-coupled receptor protein signaling pathway [78], neuron projection [79], and di- and trivalent inorganic cation transmembrane transporter activity [77, 78]. Several KEGG pathways have been previously implicated in epilepsy, including FGF signaling [59, 60], ErbB signaling [61], PTEN signaling [58], glucocorticoid receptor signaling [80], GNRH signaling [81], NGF signaling [82], and renin-angiotensin signaling [83].

On the same time, many enriched pathways are associated with cancer, such as the molecular mechanisms of cancer and prostate cancer signaling. Subsequently, nine types of tumor were observed to be significantly influenced by hsa-miR-9-5p. Specifically, upregulated miR-9-5p correlated with worse survival of seven tumor types (including ESCA, KIRC, LIHC, LUAD, SARC, STAD, and BRCA), while miR-9-5p could improve the survival of two tumor types (GBM and LGG) in the brain.

This effect has also been reported in several previous studies. Zhang et al. found that miR-9-5p suppresses the proliferation of GBM cells by targeting forkhead box P2 (FOXP2), which improves tumor survival [33].

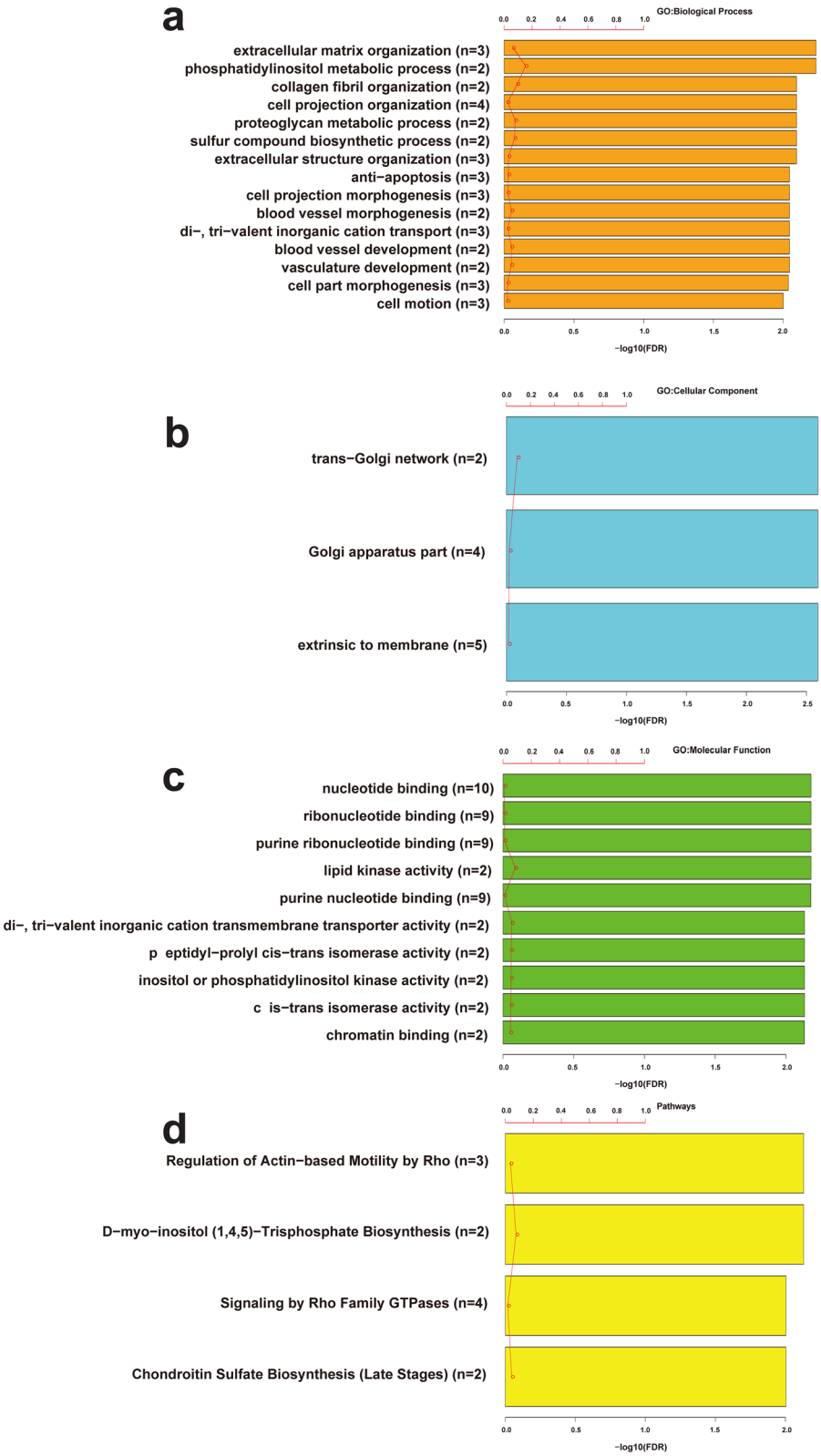

Fig. 7 Enrichment analysis of targets of hsa-miR-9-5p associated with glioma-associated epilepsy. **a** GO (BP) enrichment analysis. **b** GO (CC) enrichment analysis. **c** GO (MF) enrichment analysis. **d** KEGG enrichment analysis

Likewise, previous studies indicate that miR-9 may act as a suppressor of glioma [34–36]. This may be because that miR-9 inhibits *FOXP1* and antagonizes the tumor growth advantage granted by mutant epidermal growth factor receptor signaling [34]. In addition, hsa-miR-9 has been shown to reduce the migration and invasion of GBM cells by inhibiting the MAPKAP signaling [37]. However, contrary to our findings, Wu et al. reported that elevated miR-9 expression signals an adverse prognosis for human GBM and LGG [32]. In addition to the inconsistency of detection technique and experimental methods, a key reason for this discrepancy may be that they did not distinguish between miR-9-5p and miR-9-3p [33].

Previous evidence indicates that epilepsy in glioma improves the survival of patients with glioma [63–65], which prompted us to further investigate whether miR-9-5p can be a crucial factor in this interesting phenomenon. There may be a connection between epilepsy and tumors. The top 10 hub genes *SH3PXD2B*, *ARF6*, *ANK2*, *KIF13A*, *RAB8A*, *CCNE2*, *PHIP*, *PBRM1*, *STK38L*, and *TBL1XR1* may be the key genes involved in the induction of glioma-associated epilepsy. ARF6 is a member of the ADP ribosylation factors (ARFs) family, which controls various cellular functions in eukaryotic cells, including membrane transport and actin cytoskeleton rearrangement [84]. Previous studies have found that ARF6 knockout mice reduce GABAergic neurons in the dentate gyrus (DG) region of the hippocampus, which in turn affects the activity of the hippocampal neuronal cluster in mice, and that loss of inhibition of network activity in the DG region of the hippocampus leads to excitability [84]. And GABA neurons promote the proliferation of glioma cells [85]. Thus, miR-9-5p may reduce the density of GABAergic neurons through targeted inhibition of ARF6, leading to an excitatory/inhibitory imbalance, which may be a key mechanism for improved survival for patients with glioma-associated epilepsy. But further studies are needed to confirm this. In addition, enrichment analysis of targets of hsa-miR-9-5p associated with glioma-associated epilepsy indicates signaling by Rho family GTPases may be a key mechanism involved in miR-9-5p in regulating glioma-associated epilepsy and improving survival. The GTPases of Rho family are key regulators of actin dynamics and play a central role in mediating signal transduction from extracellular stimuli targeting the cytoskeleton [86]. Previous studies have indicated that the GTPases of Rho family are implicated in epileptic seizures and the maintenance of malignant phenotypes of glioma [86–88]. Thus the GTPase of Rho family may act as a common pathway for miR-9-5p afecting epilepsy and glioma.

Limitations

This study had some limitations. First, this was a preliminary study based on bioinformatics methods; an in-depth study on the role and mechanism of miR-9-5p in epilepsy and glioma is needed. Second, since human specimens are difficult to obtain, we only collected cortical samples, rather than hippocampal samples, which was inconsistent with the use of animal specimens. In the future, research using glioma specimens to investigate the functions and mechanisms associated with glioma and epilepsy is warranted.

Conclusions

Together, our findings demonstrate that miR-9-5p may contribute to the pathophysiology of epilepsy, and overexpression of miR-9-5p significantly exacerbates the lithium-pilocarpine-induced epileptic seizures. miR-9-5p may improve the survival of glioma patients and may underlie the phenomenon that the occurrence of epilepsy predicts better survival in glioma patients. Understanding the effects and functional mechanisms of miR-9-5p in epileptic seizures and glioma may provide a theoretical basis for the diagnosis and treatment of patients with epilepsy and glioma.

Abbreviations

ANK2: Ankyrin 2, neuronal; ARF6: ADP ribosylation factor 6; BP: Biological process; BRCA: Breast invasive carcinoma; CC: Cellular component; CCNE2: Cyclin E2; DEGs: Differentially expressed genes; EGFR: Epidermal growth factor receptor; ESCA: Esophageal carcinoma; FFPE: Formalin-fixed paraffin-embedded; FOXP1: Forkhead box p1; FOXP2: Forkhead box p2; GBM: Glioblastoma multiforme; GEO: Gene Expression Omnibus; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; KIF13A: Kinesin family member 13A; KIRC: Kidney renal clear cell carcinoma; LFPs: Local field potentials; LGG: Brain lower-grade glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; MF: Molecular function; MAPKAP: Mitogen-activated protein kinase-activated protein; PBMC: Peripheral blood mononuclear cell; PBRM1: Polybromo 1; PHIP: Pleckstrin homology domain interacting protein; PPI: Protein protein interaction; PTEN: Phosphatase and tensin homolog; rAAV: Recombination adeno-associated virus; RAB8A: Member RAS oncogene family; SARC: Sarcoma; SE: Status epilepticus; SH3PXD2B: SH3 and PX domains 2A; STAD: Stomach adenocarcinoma; STK38L: Serine/threonine kinase 38 like; TBL1XR1: Transducin (beta)-like 1 X-linked receptor 1.

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Authors' contributions

WSL performed experiments, collected data, analyzed data, and wrote the paper. XZH and NNB participated in the design of the study and data analysis. MYC, XMD, MZ, and LZ participated in data collection. SXW and GHJ participated in the design of the experiments and supervised the study. All authors approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
All animal experiments were performed following the Animal Experimentation Ethics Committee of the North Sichuan Medical College (approval number NSMC [A]2021[21]). All human specimens were randomly chosen from 220 specimens in the epileptic brain tissue bank from Chongqing Medical University and the study protocol was approved by the Ethics Committee of Chongqing Medical University. All patients or their relatives gave their written informed consent before participation.

Consent for publication
Not applicable.

Competing interests
All authors claim that there are no conflicts of interest.

Author details
[1]Department of Neurology, Affiliated Hospital of North Sichuan Medical College, No.1 Maoyuan South Road, Nanchong 637000, China. [2]Institute of Neurological Diseases, North Sichuan Medical College, Nanchong, Sichuan, China. [3]Department of Neurosurgery, Daping Hospital, Army Medical University, Daping, Chongqing, China.

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