Expression and Significance of miR-654-3p/ARL4C in Patients with Glioma

Hua Gao (✉ huagao_bj@163.com)

Capital Medical University <https://orcid.org/0000-0001-8279-1579>

Yang Zhongjin

Capital Medical University

Li Zhenye

Capital Medical University

Li Zhenzong

Capital Medical University

Research Article

Keywords: glioma, overall survival, ADP-ribosylation-like factor4C, miR-654-3p

Posted Date: August 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-687117/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: ADP-ribosylation-like factor4C (ARL4C) is overexpressed in several cancer tissues and is involved in cancer development. Increasing evidence reveals that aberrant microRNAs (miRNAs) expression play a crucial role in the tumorigenesis of cancers. Nevertheless, the exact mechanism that regulates ARL4C expression in glioma has not been fully elucidated. The aim of this study was to investigate expression and significance of miR-654-3p/ARL4C in glioma.

Methods: CGGA and TCGA databases were used to study the prognosis role of miR-654-3p, ARL4C and the relationship between ARL4C and pathological grade in gliomas. Real-time quantitative PCR was performed to detect the levels of miR-654-3p and ARL4C in glioma tissues. CCK-8 and colony formation experiments were used to observe the effects of miR-654-3p and ARL4C on the proliferation of U87 cells.

Results: CGGA database and TCGA database both showed that the level of ARL4C was increased along with the WHO grades of glioma, and patients with high ARL4C had lower IDH mutation ratio, older age and poor overall survival (OS) compared with patients with low ARL4C. The clinical features in patients with high miR-654-3p showed the opposite trend compared with patients with high ARL4C. In vitro experiments showed that overexpression of ARL4C promoted cell proliferation in U87 cells. Dual-luciferase reporter gene assays showed that ARL4C was the target gene of miR-654-3p, and miR-654-3p mimics could inhibit the cell proliferation of U87 cells, and ARL4C partly counteracted the role of miR-654-3p in U87 cells.

Conclusions: miR-654-3p/ARL4C is associated with pathological grade of glioma, and patients with high ARL4C or low miR-654-3p have shorter OS. miR-654-3p/ARL4C may serve as a prognostic factor in patients with glioma and new potential therapeutic target.

Background

Glioma is one of the commonest malignant tumors in adults, and glioblastoma multiforme (GBM) is known for its invasive and aggressive behavior with a 5-year relative survival of ~5%[1, 2]. GBM is widespread and diffusely infiltrating, typically invades multiple lobes according to elegant pathologic and radiologic study. Therefore, it is a worldwide problem of how to balance the benefits of total resection and true risks. Integration and analysis of genome-scale data from gliomas have also produced new evidence about risk and prognosis[3]. Genome-wide association studies have identified heritable risk alleles including O6-methylguanine-DNA methyltransferase methylation (MGMT), isocitrate dehydrogenase (IDH) mutation, and a glioma cytosine–phosphate–guanine island methylator phenotype[4–6]. The accumulating data in understanding glioma heterogeneity revealing a relatively small number of etiologically similar glioma subtypes that can be characterized by tumor biomarkers[7].

ADP-ribosylation factor (ARF)-like (ARL) 4C (ARL4C) is a member of the ARF small GTP-binding protein subfamily, and is involved in vesicle transport, membrane transport, organelle structure and cytoskeleton remodeling function[8–10]. ARL4C is frequently overexpressed in hepatocellular carcinoma, colorectal

cancer, lung cancer, and tongue cancer but little in normal regions of these tissues[11, 12]. Three gastric cancer datasets showed that ARL4C was a poor prognostic factor, and promoted cell proliferation and invasive capacity through activating epithelial-mesenchymal transition and motility. There was a significant association with shorter survivals in high ARL4C patients with renal cell carcinoma[13, 14]. Except Wnt- β -catenin and growth factor-Ras signaling pathway[15], AKT pathway may regulate the level of ARL4C in lung cancer cells, and chemotherapy drug hydroxycamptothecin could reduce the levels of ARL4C by inhibiting the activation of the AKT pathway[16]. Chemically modified antisense oligonucleotides (ASO) targeting ARL4C effectively inhibited cell proliferation and migration in hepatocellular carcinoma cells and colorectal cancer cells in vitro experiments[11].

MicroRNAs (miRNAs) can contribute to cancer development and progression through binding to the 3'-untranslated regions of target mRNA, and cause blockage of translation or mRNA degradation[17]. In this study, we measured the levels of ARL4C and its role in glioma prognosis using data from The Cancer Genome Atlas (TCGA), Chinese Glioma Genome Atlas (CGGA) databases, and verified the tumorigenesis role of ARL4C using 45 clinical samples. Through TargetScan, miRWalk, miRDB, miRTarBase and sarBase databases, we filtered and further proved the inhibition role of miR-654-3p in glioma targeting ARL4C.

Material And Methods

Patients and samples

The transcriptome data and clinical information of 321 patients from CGGA database (<http://www.cgga.org.cn/>) and 609 patients from TCGA database (<https://tcga-data.nci.nih.gov/tcga/>) was used. CGGA RNA sequencing database and microarray were used as validation set. 45 samples from Beijing Tiantan Hospital from August 2013 to December 2017 were used for further verification. The study was approved by the institutional review board of Beijing Tiantan Hospital affiliated to Capital Medical University (KY2019-143-02), and all patients or legal representatives signed an informed consent form.

Immunohistochemistry staining

45 formalin-fixed paraffin-embedded tissue blocks were sectioned. The slide was cut into 4- μ m sections and incubated with anti-ARL4C (rabbit polyclonal, 1:300, ab122025, Abcam) primary antibodies. H-score was obtained by multiplying the staining intensity by a constant to adjust the mean to the strongest intensity [H-score = 3 $\times$ (percentage of strong staining)] (1.0%, weak; 2.0%, moderate; 3.0%, strong) to give a score ranging from 0 to 300.

Cell proliferation and colony formation experiment

U87-MG cells were adjusted to a density of 1×10^5 cells/ml. A total of 100 μ l of the cell suspension was plated into each well of a 96-well plate and cultured overnight. After transient infection with 3 μ g vector or ARL4C or miRNA-654-3p plasmid on 1×10^6 cells using Lipo3000 for 24 h, 48 h, and 72 h, 20 μ l CCK8 solution was added to each well, and the cultures were further incubated for 4 h. Absorbance was measured at 450 nm using an ELISA plate reader (Thermo, USA).

1×10^3 U87-MG cells were seeded into a 6-well culture plate. After adherence, cells were transfected with ARL4C or miRNA-654-3p plasmid. Cells were maintained in a 37°C, 5% CO₂ incubator for 2 weeks. To maintain transfection efficiency in the whole experiment, transfection was repeated at the 7th day after the first transfection. Cell colonies were stained with crystal violet (Zhongshan Company, Beijing, China) and photographed at the end of after 2 weeks of incubation.

Reverse transcription and quantitative PCR

Microarray hybridization and qRT-PCR were performed as previously described[18]. Total RNA of 30 samples was extracted and purified using the Rneasy®Mini Kit (QiaGen, Hilden, Germany) following manufacturer's instructions. RT-qPCR was performed on a QuantStudio5 (Applied biosystems, Singapore). The fold-change in differential expression for each gene was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$ method) in R package with "PCR" functions (<https://github.com/MahShaaban/pcr>), a GAPDH reference gene, and the "1/2a" stage reference group. The primers of genes were listed as following: ARL4C F: atgcatttcccagaaccgtg, R: ttctgatgacccaaaccacca; GAPDH F: aggtcggagtgcaacggattt, R: tgacaagcttcccggttctca. miRNA-654-3p F: AGGTGACCAAGTTCGCCGAG, R: GACGTGATAGGTGGTGGCCG. U6 F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT.

SDS-PAGE and western blot analyses

Samples (up to 10 mg) were lysed in lysis buffer containing 1% Nonidet P-40 (Calbiochem, Merk, Darmstadt, Germany) and protease and phosphatase inhibitor cocktails (Roche, IL, USA) overnight at 4 °C. Total extracts were centrifuged at 12,000 g for 30 min at 4 °C, and protein concentration was determined with the BCA method (Pierce Biotechnology, IL, USA). A total of 40 μ g of protein per lane was loaded onto 10% Bis-Tris SDS-PAGE gels, separated electrophoretically, and blotted onto polyvinylidene fluoride membranes (Merk). The blots were incubated with antibodies against anti-ARL4C (1:2,000) and anti-GAPDH (1:8000, ab181602) followed by a secondary antibody (1:10,000) tagged with horseradish peroxidase (Santa Cruz Biotechnology). Blots were visualized by enhanced chemiluminescence, and densitometry was performed using a fluorescence image analyzer (Amersham Imager 600, GE, MA, USA). GAPDH was used as a loading control.

Dual-luciferase reporter gene assay

The miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/php/index.php>) was used to predict the target mRNAs of miRNAs. ARL4C sequences were acquired from Ensembl (<http://asia.ensembl.org/index.html>). Interacting miRNA and mRNA sequences were analyzed by Global Align in Blast (<https://blast.ncbi.nlm.nih.gov>). JAG1 sequences harboring either the wild-type (WT) or mutated binding sites (MT) for miR-654-3p were amplified by PCR and cloned into the pmirGLO luciferase vector (Promega, Madison, WI, USA) to create wild-type or mutant pmirGLO-ARL4C vectors, respectively, using the Lipofectamine 3000 transfection kit (Promega, Madison, WI, USA). U87-MG and C6 cells harboring the wild-type or mutant pmirGLO-ARL4C were co-transfected with the mimics or miR-NC, respectively, for 48 h. After transfection, luciferase reporter gene activity was determined following the instructions of the dual-luciferase detection kit (Promega, Madison, WI, USA).

Receiver operating characteristic (ROC) analysis

ROC curve analysis was performed using the ROCR package of the free software R (<https://www.r-project.org/>). The 95% confidence interval for area under the curve (AUC) was calculated according to Hanley and McNeil[19].

Statistical analysis

All statistical analyses were conducted using SPSS Statistics Version22 (IBM Corporation, Armonk, New York, USA). An unpaired Student's test and a chi-square (Fisher's exact) test were used to compare quantitative and qualitative data. P-value of less than 0.05 was considered significant.

Results

ARL4C expression is associated with World health organization (WHO) grade and age

Firstly, we compared the expression level of ARL4C in 609 glioma patients from TCGA database and 321 glioma patients from CGGA database according to WHO grade. Data from TCGA database showed that the values of ARL4C were 9.18 ± 0.07 in WHO II patients, and 9.82 ± 0.08 in WHO III patients, and 11.18 ± 0.08 in WHO IV patients ($F=140.8$, $P<0.001$) in Figure 1A. Similarly, CGGA database showed the values of ARL4C were 10.67 ± 1.32 in WHO II patients, and 23.34 ± 2.28 in WHO III patients, and 34.18 ± 2.1 in WHO IV patients ($F=38.48$, $P<0.001$) in Figure 1B. The pictures of ARL4C were shown in Supplementary Figure 1. There were higher values of ARL4C in elder patients according to TCGA database ($r=0.459$, $P<0.001$) and CGGA database ($r=0.234$, $P<0.001$) in Figure 1CD. Patients with IDH wild-type showed higher ARL4C than those with mutant-type in TCGA database (11.09 ± 0.06 vs. 9.18 ± 0.05 ; $t=22.83$, $P<0.001$) and CGGA database in Figure 1EF (34.95 ± 1.88 vs. 14.84 ± 1.39 ; $t=8.769$, $P<0.001$). Meanwhile, there was the higher ARL4C levels in patients with non-codeletion 1p19q compared with

patients with codeletion 1p19q between patients with non-codeletion and patients with codeletion ($P<0.001$) in Figure 1GH.

The patients were divided into patients with high ARL4C (high ARL4C group) and patients with low ARL4C (low ARL4C group) according to the median of TPM value of ARL4C. There was poorer overall survival (OS) in high ARL4C group than low ARL4C group based on TCGA database (15.6 ± 1.15 vs. 27.77 ± 1.87 , $P=0.000$) and CGGA database (27.02 ± 2.81 vs. 70.67 ± 4.18 , $P=0.000$) in table 1. There were older patients, more WHO IV, more wild type IDH chance and non-Codeletion chance in high ARL4C group compared with low ARL4C group based on two databases as shown in table 1.

Table 1

Association between ARL4C expression and clinico-pathological characteristics

Variables	TCGA			CGGA		
	High ARL4C	Low ARL4C	<i>P</i> value	High ARL4C	Low ARL4C	<i>P</i> value
Age (years)	53.73±0.86	40.95±0.73	0	45.88±0.99	40.01±0.84	0
Gender						
Male	180	174	0.467	107	92	0.098
Female	124	131		54	68	
WHO Grade						
II	49	167	0	15	88	0
III	115	126		40	39	
IV	140	12		106	33	
OS (month)	15.6±1.15	27.77±1.87	0	27.02±2.81	70.67±4.18	0
IDH						
WT	207	18	0	117	29	0
Mu	92	286		44	130	
NA	5	1		0	1	
1p19q						
Codel	41	110	0	8	59	0
non-Codel	259	194		151	98	
NA	4	1		2	3	

Patients with high ARL4C predict poor prognosis

Follow-up time was 21.67±1.12 months in TCGA database, and 48.37±1.77 months in CGGA database. Logrank analysis showed that patients with high ARL4C had poor prognosis compared with patients with low ARL4C based on two database in Figure 2AB ($P<0.001$). Then, we evaluated the correlation of clinical features and prognosis by Kaplan-Meier (K-M) survival curve analysis with the data from the TCGA microarray database and CGGA RNA sequencing database, respectively. Univariate Cox regression analysis showed that the overall survival (OS) time was related to age, WHO grade, IDH mutation state, 1p19q deletion state and ARL4C in table 2 and table 3. Multivariate analysis further confirmed that ARL4C was the independent risk factor for patients with glioma. ROC curve were 0.741 in TCGA database and 0.713 in CGGA database as shown in Figure 2CD.

Table 2

Univariate and multivariate Cox regression analysis of prognosis in 609 glioma patients from TCGA database

<i>Features</i>	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Age	1.026	1.017-1.036	.000	—	—	—
Grade	4.993	3.875-6.434	.000	3.267	2.462-4.334	.000
IDH	1	0.999-1.000	0.012	—	—	—
1p19q	4.54	2.670-7.722	.000	2.040	1.164-3.576	.013
ARL4C	5.985	4.173-8.585	.000	2.511	1.671-3.775	.000

Table 3

Univariate and multivariate Cox regression analysis of prognosis in 325 glioma patients from CGGA database

<i>Features</i>	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Age	1.033	1.02-1.046	.000	—	—	—
Grade	2.873	2.385-3.46	.000	2.399	1.992-2.889	.000
IDH	0.355	0.268-0.471	.000	—	—	—
1p19q	5.886	3.607-9.604	.000	3.72	2.24-6.179	.000
ARL4C	1.013	1.009-1.017	.000	—	—	—

ARL4C promoted cell proliferation in U87-MG cell line

To testify the role of ARL4C in glioma, we constructed the plasmid of ARL4C. And RT-PCR and Western blot experiments both showed overexpression of ARL4C in U87-MG in Figure 3AB. Cell proliferation showed the cell viability increased in the time-manner after overexpression of ARL4C in Figure 3C. The OD values of control group in U87-MG cells were 0.65 ± 0.03 , 0.91 ± 0.05 and 1.31 ± 0.06 after 24h, 48h and 72h culture. The OD values of ARL4C group were 0.75 ± 0.04 , 1.25 ± 0.06 and 1.97 ± 0.09 in Figure 3C, respectively ($P < 0.05$). There was no statistical difference between control group and vector group. Colony forming experiment showed that there was more cell colony in ARL4C group compared with control group or vector group in Figure 3D ($P < 0.01$).

ARL4C was the target gene of MiR-654-3p in glioma

Through TargetScan, miRWalk, miRDB, miRTarBase and sarBase databases, miR-654-3p was the candidate miRNA of ARL4C in Figure 4A. Dual luciferase reporter gene experiment showed ARL4C was the target gene of miR-645-3p in Figure 4B. RT-PCR and Western blot experiment showed the levels of ARL4C were relieved by overexpression of miR-654-3, and can be rescued by overexpression of ARL4C again in Figure 4CD. Cell viability and colony forming experiment showed the inhibition role of miR-654-3p, and overexpression of ARL4C partly counteracted the role of miR-654-3p in U87-MG cells in Figure 4EF.

Patients with low miR-654-3p predict poor prognosis

Follow-up time was 54.21 ± 4.23 months on 198 microRNA assay for CGGA database. We analysed the relationship of miR-654-3p and clinical features based on 198 microRNA assay from CGGA database. The TPM value of miR-654-3p was declined by WHO grade in Figure 5A ($F = 40.79$, $P < 0.001$). There was a negative correlation between miR-654-3p levels and age in Figure 5B ($r = -0.289$, $P > 0.001$). The levels of miR-654-3p in patients with IDH WT were lower than patients with IDH mutation in Figure 5C. These

patients were divided into low miR-654-3p group and high miR-654-3p according to the median. Low miR-654-3p group had poor OS than high miR-654-3p in Figure 5D (HR: 1.581, 95%CI: 1.089-2.296, $P=0.016$). To verify the relationship between ARL4C and miR-654-3p, we analyzed the mRNA levels of ARL4C and miR-654-3p in 45 patients with glioma. The correlation coefficient was -0.475 between Δ CT value of miR-654-3p and Δ CT value of ARL4C in Figure 5E.

Discussion

Glioma is the most common malignant primary brain tumor, and glioblastoma is prone to drug resistance to chemotherapy drugs as the highest malignancy[20]. Over the past decade, gene mutations associated with epigenetic regulation have been identified as the key drivers of glioma subtypes with unique clinical characteristics. According to the 2016 WHO glioma classification guidelines, the corresponding genetic changes were used as the classification criteria to guide the clinical treatment of brain glioma[7]. In this study, we found that ARL4C is associated with pathological grade of glioma, and patients with high ARL4C had shorter OS than patients with low ARL4C. miR-654-3p played an important role in the tumorigenesis of glioma by affecting the levels of ARL4C.

ARL4 family proteins, including ARL4A, ARL4C and ARL4D, all have a nuclear localization signal and abnormally elevated guanine nucleotide expression, which is related to cell diffusion and migration function[21]. ARL4C is composed of 192 amino acids with the GTP-enzyme activity, and participates in a variety of physiological progresses in vivo, for example cholesterol secretion, vesicle transport and cell migration, etc[22]. In vitro and in vivo experiments confirmed that knockdown of ARL4C inhibited proliferation and osteogenesis by regulating the Wnt pathway in human adipose derived stem cells[23]. It also reported that ARL4C interacted with α tubulin and affected the transport of intracellular vesicles, and ARL4C regulated the migration of ovarian cancer cells rather than regulating their growth[24]. Studies showed that knockdown of ARL4c relieved cell invasion or migration ability, Slug expression level and filopodia formation in gastric cancer AGS and 58AS9 cells[13].

Based on CGGA database and TCGA database, ARL4C was rich in glioma, furthermore, the levels of ARL4C were increased with the WHO grades. Univariate and multivariate Cox analysis of TCGA database both showed that ARL4C, IDH mutation, 1p19q mutation were independent factors for poor prognosis in patients with glioma. Patients with high ARL4C had less IDH mutation and more elderly patients compared with patients with low ARL4C. And follow-up data showed the levels of ARL4C was associated with the malignant progression of glioma, including recurrence and death. Our data suggested that ARL4C was a biomarker for poor prognosis in glioma, which was consistent with renal cell carcinoma[12]. RT-PCR experiment further confirmed that the levels of ARL4C were positive correlation with glioma WHO grades. We speculated that ARL4C was not an independent factor in 45 patients or CGGA database, which was caused by small size or more patients with glioblastoma in CGGA database.

The antisense oligonucleotides could inhibit the cell proliferation and migration in hepatocellular carcinoma and colorectal cancer cells through targeting ARL4C, and delayed the progression whether

primary tumor or metastatic colorectal cancer in immunodeficient mice[11]. In vivo experiments showed that transbronchial application of ARL4C ASO-1316 inhibited the formation of orthotopic bronchial tumor induced by lung cancer cell lines[25]. Our analysis indicates that ARL4C mRNA is overexpressed in glioma, hence, miRNAs' regulation on ARL4C is further investigated. We screen out miR-654-3p as the promising miRNAs which might target ARL4C in glioma. miR-654-3p plays various biological roles in diverse non-small cell lung cancer by targeting specific genes[26]. Moreover, they could participate in regulating several hallmarks of cancer, such as proliferation, cell death and senescence, angiogenesis, invasion and metastasis[27, 28]. CGGA database showed that the levels of miR-654-3p decreased with the increase of glioma grade. In fact, CGGA database showed patients with low miR-654-3p had shorter OS than patients with high ARL4C. In vitro functions also confirmed the tumor suppressor role of miR-654-3p in U87 cells. In this study, RT-PCR experiment showed the negative correlation of ARL4C and miR-654-3p. Dual luciferase reporter gene experiment verified that ARL4C was one of target genes of miR-654-3p.

Conclusion

In all, our work is of significance for comprehensively understanding the mechanisms of ARL4C in glioma. Our data demonstrated that ARL4C was an independent factor for poor prognosis, and miR-654-3p could function as upstream modulators of ARL4C in glioma cells. Our study provides more comprehensive insights into the specific role of ARL4C that benefits the development of novel strategies for glioma treatment, in particular, ARL4C may be a valuable new target for glioma treatment in wild-type IDH patients.

Abbreviations

ADP-ribosylation factor (ARF)-like (ARL) 4C, ARL4C; Antisense oligonucleotides, ASO; Area under the curve, AUC; Chinese Glioma Genome Atlas, CGGA; Glioblastoma multiforme, GBM; Isocitrate dehydrogenase, IDH; O6-methylguanine-DNA methyltransferase methylation, MGMT; MicroRNAs, miRNAs; Mutated binding sites, MT; The Cancer Genome Atlas, TCGA; Wild-type, WT; World health organization, WHO;

Declarations

Ethics approval and consent to participate

The study protocols were approved by the Internal Review Board of Beijing Tiantan Hospital, which was affiliated to Capital Medical University, and conformed to the ethical guidelines of the Declaration of Helsinki (No. KY2016-035-01).

Consent for publication:

We certify that we have participated sufficiently in the work to take public responsibility for the appropriateness of the collection, analysis, and interpretation of the data. We have reviewed the final version of the manuscript and approve it for publication. This manuscript has not been published in whole or in part nor is it being considered for publication elsewhere.

Availability of data and materials

All the data generated or analyzed in this study are included in this published article and its Additional files.

Conflict of interest

The authors declare that they have no competing interests.

Funding:

This study was financially supported by the National Natural Science Foundation of China (81771489), the Natural Science Foundation of Beijing (7162035).

Authors contributions:

Yang zhongjin, Writing and methodology; Li Zhenzong, Methodology; Gao Hua, Data and follow up; Li Zhenye , Funding Acquisition and Editing

Acknowledgements:

The authors thank the laboratory technicians, data collectors, and medical editors.

References

1. Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, et al. The epidemiology of glioma in adults: a “state of the science” review. *Neurooncology*. 2014;16:896–913.
2. Jones J, Nguyen H, Drummond K, Morokoff A. Circulating Biomarkers for Glioma: A Review. *Neurosurgery*. 2021;88:E221–30.
3. Riddick G, Fine HA. Integration and analysis of genome-scale data from gliomas. *Nat Rev Neurol*. 2011;7:439–50.
4. Bell EH, Pugh SL, McElroy JP, Gilbert MR, Mehta M, Klimowicz AC, et al. Molecular-Based Recursive Partitioning Analysis Model for Glioblastoma in the Temozolomide Era: A Correlative Analysis Based

- on NRG Oncology RTOG 0525. *JAMA Oncol.* 2017;3:784–92.
5. Miller JJ, Shih HA, Andronesi OC, Cahill DP. Isocitrate dehydrogenase-mutant glioma: Evolving clinical and therapeutic implications. *Cancer.* 2017;123:4535–46.
 6. Malta TM, de Souza CF, Sabedot TS, Silva TC, Mosella MS, Kalkanis SN, et al. Glioma CpG island methylator phenotype (G-CIMP): biological and clinical implications. *Neuro Oncol.* 2018;20:608–20.
 7. Reifenberger G, Wirsching H-G, Knobbe-Thomsen CB, Weller M. Advances in the molecular genetics of gliomas - implications for classification and therapy. *Nat Rev Clin Oncol.* 2017;14:434–52.
 8. Jacobs S, Schilf C, Fliegert F, Koling S, Weber Y, Schürmann A, et al. ADP-ribosylation factor (ARF)-like 4, 6, and 7 represent a subgroup of the ARF family characterization by rapid nucleotide exchange and a nuclear localization signal. *FEBS Lett.* 1999;456:384–8.
 9. Sedgwick A, Olivia Balmert M, D’Souza-Schorey C. The formation of giant plasma membrane vesicles enable new insights into the regulation of cholesterol efflux. *Exp Cell Res.* 2018;365:194–207.
 10. Li C-C, Wu T-S, Huang C-F, Jang L-T, Liu Y-T, You S-T, et al. GTP-binding-defective ARL4D alters mitochondrial morphology and membrane potential. *PLoS ONE.* 2012;7:e43552.
 11. Harada T, Matsumoto S, Hirota S, Kimura H, Fujii S, Kasahara Y, et al. Chemically Modified Antisense Oligonucleotide Against ARL4C Inhibits Primary and Metastatic Liver Tumor Growth. *Mol Cancer Ther.* 2019;18:602–12.
 12. Fujii S, Matsumoto S, Nojima S, Morii E, Kikuchi A. Arl4c expression in colorectal and lung cancers promotes tumorigenesis and may represent a novel therapeutic target. *Oncogene.* 2015;34:4834–44.
 13. Hu Q, Masuda T, Sato K, Tobo T, Nambara S, Kidogami S, et al. Identification of ARL4C as a Peritoneal Dissemination-Associated Gene and Its Clinical Significance in Gastric Cancer. *Ann Surg Oncol.* 2018;25:745–53.
 14. Isono T, Chano T, Yoshida T, Makino A, Ishida S, Suzaki M, et al. ADP-ribosylation factor-like 4C is a predictive biomarker of poor prognosis in patients with renal cell carcinoma. *Am J Cancer Res* [Internet]. 2019 [cited 2020 Aug 27];9:415–23. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6405968/>.
 15. Matsumoto S, Fujii S, Kikuchi A. Arl4c is a key regulator of tubulogenesis and tumourigenesis as a target gene of Wnt- β -catenin and growth factor-Ras signalling. *J Biochem.* 2017;161:27–35.
 16. Sun Y, Zhang Z, Xiang F, Zhang M, Chen Q, Tang L, et al. Decreasing Arl4c expression by inhibition of AKT signal in human lung adenocarcinoma cells. *Life Sci.* 2020;246:117428.
 17. Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA.* 2006;103:2257–61.
 18. Liu C, Gao H, Cao L, Gui S, Liu Q, Li C, et al. The role of FSCN1 in migration and invasion of pituitary adenomas. *Mol Cell Endocrinol.* 2016;419:217–24.
 19. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology.* 1982;143:29–36.

20. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X, et al. CGCG clinical practice guidelines for the management of adult diffuse gliomas. *Cancer Lett.* 2016;375:263–73.
21. Hofmann I, Thompson A, Sanderson CM, Munro S. The Arl4 family of small G proteins can recruit the cytohesin Arf6 exchange factors to the plasma membrane. *Curr Biol.* 2007;17:711–6.
22. Pasqualato S, Renault L, Cherfils J. Arf. Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for “front-back” communication. *EMBO Rep.* 2002;3:1035–41.
23. Wang W, Wang S, Liu X, Gu R, Zhu Y, Zhang P, et al. Knockdown of ARL4C inhibits osteogenic differentiation of human adipose-derived stem cells through disruption of the Wnt signaling pathway. *Biochem Biophys Res Commun.* 2018;497:256–63.
24. Su D, Katsaros D, Xu S, Xu H, Gao Y, Biglia N, et al. ADP-ribosylation factor-like 4C (ARL4C), a novel ovarian cancer metastasis suppressor, identified by integrated genomics. *Am J Transl Res.* 2015;7:242–56.
25. Kimura K, Matsumoto S, Harada T, Morii E, Nagatomo I, Shintani Y, et al. ARL4C is associated with initiation and progression of lung adenocarcinoma and represents a therapeutic target. *Cancer Sci.* 2020;111:951–61.
26. Pu J-T, Hu Z, Zhang D-G, Zhang T, He K-M, Dai T-Y. MiR-654-3p Suppresses Non-Small Cell Lung Cancer Tumourigenesis by Inhibiting PLK4. *Onco Targets Ther.* 2020;13:7997–8008.
27. Geraldo MV, Nakaya HI, Kimura ET. Down-regulation of 14q32-encoded miRNAs and tumor suppressor role for miR-654-3p in papillary thyroid cancer. *Oncotarget.* 2017;8:9597–607.
28. Zhang H, Shen Z, Zhou Y, Zhang Z, Wang Q, Zhang M, et al. Downregulation of miR-654-3p in Colorectal Cancer Indicates Poor Prognosis and Promotes Cell Proliferation and Invasion by Targeting SRC. *Front Genet.* 2020;11:577948.

Figures

Figure 1

The relationship between ARL4C and pathological features in patients with glioma. The levels of ARL4C among patients with different WHO grades based on TCGA database (A) and CGGA database (B). There were positive correlation between ARL4C and age based on TCGA database (C) and CGGA database (D). There were higher ARL4C level in patients with IDH wildtype (WT) compared with patients with IDH mutation (Mu) based on TCGA database (E) and CGGA database (F). There were higher ARL4C level in patients with 1p19q non-codeletion (non-codel) compared with patients with 1p19q codeletion (codel) based on TCGA database (G) and CGGA database (H).

Figure 2

The levels of ARL4C were related to OS of patients with glioma. Follow-up data showed patients with high ARL4C had poor OS compared with low ARL4C based on TCGA database (A) and CGGA database (B). ROC curve showed the levels of ARL4C were related to death censors based on TCGA database (C) and CGGA database (D).

Figure 3

ARL4C promoted the cell proliferation in U87 cells. RT-PCR experiment (A) and Western blot experiment (B) both showed overexpression of ARL4C in U87 cells. Overexpression of ARL4C increased the cell viability of U87 cells in the time-manner (C). Colony formation experiment showed overexpression of ARL4C increased the colonies of U87 (D). * compared with control group $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Figure 4

ARL4C was the target gene of miR-654-3p. Five databases showed the target genes of miR-654-3p (A). Double luciferase reporter gene experiment showed luciferase activity in co-transfected with ARL4C-WT plasmid and miR-654-3p mimic was significantly lower than that of other groups (B). RT-PCR experiment (C) and Western blot experiment (D) both showed that overexpression of miR-654-3p could relieve the levels of ARL4C in U87 cells. Overexpression of miR-654-3p inhibited the cell viability of U87 cells in the time-manner (E). Colony formation experiment showed overexpression of miR-654-3p reduced the colonies of U87 (F). * compared with control group $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Figure 5

The levels of miR-654-3p were related to OS of patients with glioma. Follow-up data showed patients with low miR-654-3p had poor OS compared with high miR-654-3p based on CGGA database (A). There were negative correlation between miR-654-3p and age based on CGGA database (B). There were higher miR-654-3p level in patients with IDH Mu compared with patients with IDH WT based on CGGA database (C). Follow-up data showed patients with low miR-654-3p had poor OS compared with high Mir-654-3p based CGGA database (D). There was negative correlation between ARL4C and miR-654-3p (E).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementaryFigure1.tif](#)