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Original Article

Long noncoding RNA lysophospholipase-like 1-2 as ceRNA modulates glioma metastasis by regulating miR-217/YWHAG

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Abstract: Background: LncRNA-LYPLAL1-2 (lysophospholipase-like 1-2) is expressed at a very low level in gliomas, which are some of the most aggressive tumors. However, the function and mechanism of LYPLAL1-2 are not clear. The purpose of this study was to explore the role of LYPLAL1-2 in glioma. Methods: Reverse transcription and quantitative PCR (qRT-PCR) was used to quantify the levels of lncRNA-LYPLAL1-2, miR-127, and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (YWHAG) in glioma tumor tissue and cells. Transwell assays were used to determine the migratory capacity and invasiveness of glioma cells. Hematoxylin and eosin staining was used to identify the metastatic capacity of glioma cells transfected with lncRNA-LYPLAL1-2 *in vivo*. Western blot analysis was used to identify the levels of YWHAG and related proteins. Luciferase reporter assay was used to identify whether miR-217 is the direct target of lncRNA-LYPLAL1-2. Results: LncRNA-LYPLAL1-2 was significantly downregulated in glioma tumor tissue. LncRNA-LYPLAL1-2 overexpression suppressed migration and invasion *in vitro* and *in vivo*. LncRNA-LYPLAL1-2 acted as a sponge molecule and targeted miR-217 in glioma cells. YWHAG was identified as the target gene of miR-217 and was indirectly regulated by lncRNA-LYPLAL1-2. Conclusions: LncRNA-LYPLAL1-2 suppressed glioma metastasis via the miR-217/YWHAG axis and is expected to be a potential target for early diagnosis and treatment of gliomas.

Keywords: Glioma, lncRNA-LYPLAL1-2, metastasis, ceRNA

Introduction

Glioma is the most common central nervous system (CNS) tumor, accounting for approximately 80% of CNS tumors [1]. It is an aggressive primary brain tumor and poorly diagnosed. Currently, the main therapies for glioma include surgery (maximal tumor resection), combined with radiotherapy and chemotherapy (e.g., temozolomide), both of which are partly effective [2-4]. Moreover, the outcomes of most patients with glioma are unsatisfactory, due to the high rates of recurrence and metastasis. It is reported that the median survival after treatment ranges between 12 and 15 months, and only 3-5% of patients survive for more than 5 years after diagnosed [1, 5], the lowest 5-year survival rate among all cancers [6]. Hence, it is of great urgency to investigate the mechanisms of occurrence and development in glioma, which could eventually benefit the development of novel biomarkers for early diagnosis as well as effective therapeutic targets.

Long non-coding RNAs (lncRNAs) consist of more than 200 nucleotides (nts) [7]. LncRNAs mainly act as molecular sponges to prevent microRNAs from binding to their mRNA targets and mediating their functions [8-10]. LncRNAs are getting increasing attention from researchers due to their broad range of functions, from modulating gene abundance to protein translation and stability [11, 12]. Recently, lncRNAs have frequently been reported to be involved in the onset of cancers, including glioma [13, 14]. Several lncRNAs have already been reported to act as key mediators in glioma onset and progression; for example, NEAT1 promotes glioma cell proliferation and invasion by binding to EZH2 and catalyzing histone H3 lysine 27 tri-

methylation (H3K27me3) on target promoters [7]. Previous studies have shown that lncRNA lysophospholipase-like 1-2 (lncRNA-YPLAL1-2) is expressed at a low level in gliomas, and was found to be downregulated in pancreatic ductal adenocarcinoma compared to the adjacent noncancerous tissue [15, 16]. Moreover, lncRNA-LYPLAL1-2 has been reported to be expressed at a lower level in recurring gliomas compared with primary gliomas [16]. However, the exact roles of lncRNA-LYPLAL1-2 in glioma have not yet been investigated.

MicroRNAs (miRNAs), small noncoding RNAs, can negatively regulate downstream target gene expression by binding to the complementary sequences at the 3'-UTR of mRNA [17, 18]. MiR-217 has been found to promote the proliferation and invasion of glioblastoma by repressing its target gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma (YWHAG) [19]. Recent studies also demonstrated that lncRNAs can function as competing endogenous RNAs (ceRNAs) by sponging miRNAs to regulate gene expression at the post-transcriptional level [20]. However, to the best of our knowledge, whether lncRNA-LYPLAL1-2 is involved in the development and progression of glioma by sponging miR-217 has not yet been identified.

In our study, we examine the abundance of lncRNA-LYPLAL1-2 in glioma and the role of lncRNA-LYPLAL1-2 in glioma recurrence, aiming to provide novel ideas for developing effective therapies for glioma.

Materials and methods

Patients and samples

Glioma samples and corresponding adjacent noncancerous tissue was collected from 55 glioma patients during gross total resection surgery under a microscope. Glioma patients undergoing tumor resection surgery in Wenzhou People's Hospital from September 27, 2013 to February 20, 2015 were enrolled in the study. All patients provided written informed consent. All glioma tumors were confirmed by histopathology and classified into early stage (I/II) and advanced stage (III/IV) according to the World Health Organization (WHO) classification criteria for glioma tumors [21]. The study was approved by the ethics committee of Wenzhou People's Hospital.

Glioma animal model construction

All animal studies were performed under the protocol approved by the Animal Research Committee of Wenzhou People's Hospital in strict accordance with the "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals". In our study, 4-6-week-old BALB/c mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Glioma cells (U251 and U87) were transfected with OEC (overexpression control, pcDNA3.1) or OE-LYPLAL1-2 (pcDNA3.1-lncRNA-LYPLAL1-2), and then suspended in a mixture of phosphate-buffered saline (PBS) and Matrigel matrix basement membrane extract (BD Biosciences, San Jose, CA, USA). The cell suspensions (8×10^6 cells/100 μL/mouse) were injected into the subcutaneous. Thirty days after injection mice were sacrificed, and lungs and livers were collected for H&E staining.

Cell lines and culture

Human glioma cell lines (U87, U251), astrocytes, and HEK-293T cells were obtained from the Chinese Academy of Sciences (Shanghai, China). U87 and U251 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified atmosphere of 5% CO_2 at 37°C.

RNA isolation, reverse transcription, and quantitative PCR (qRT-PCR)

Total RNA was isolated from glioma tumor tissuses or glioma cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was produced using a reverse transcript kit from Takara (Dalian, China). qRT-PCR was conducted using the SYBR Green Real-Time PCR Kit (Qiagen, Hilden, Germany). Data were analyzed by the 2-ΔΔCT method. The data are presented as the fold change in mRNA/miRNA level normalized against β-actin gene or U6 snRNA level. Sequences of primers used for qRT-PCR analysis are shown in **Table 1**.

Cell transfection

The miR-217 mimic and its negative control (miR-NC) were obtained from RiboBio (Guangzhou, China). The full-length lncRNA-LYPLAL1-2

Table 1. Primer sequences for qRT-PCR

Gene	Primer	Sequence
MiR-217	qF	5'-TACTCAACTCACTACTGCATCAGGA-3'
	qR	5'-TATGGTTGTTCTGCTCTCTGTGTC-3'
YWHAG	qF	5'-GCCGTATGTCAGGATGT-3'
	qR	5'-GCCAGGTAGCGGTAAT-3'
U6	qF	5'-CTCGCTTCGGCAGCACA-3'
	qR	5'-AACGCTTCACGAATTTGCGT-3'
β-actin	qF	5'-ACCGAGCGCGGCTACAG-3'
	qR	5'-CTTAATGTCACGCACGATTTCC-3'
LncRNA-LYPLAL1-2	qF	CTCACCAGCTGTGGCCTTTTA
	qR	CTGCTCCCCCTTTGCATGT

F, forward primer; R, reverse primer.

(wild type, WT; or mutant, Mut) was synthesized and cloned into vector psiCHECK2 (Promega, Madison, WI, USA). Transfection of cells with siRNA was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiencies were detected by qRT-PCR at 48 h post transfection.

Transwell assay of migration and invasion

U251 and U87 cells transfected with LYPLAL1-2 and miR-NC/miR-217 were suspended in serum-free RPMI 1640 at a density of 5×10^5 cells/ml. Cell suspension (100 μl) was seeded on the upper 8-μm-pore-size insert of a 24-well Transwell chamber (Corning), and RPMI 1640 supplemented with 10% FBS (500 μl) was added to the lower chamber. Cells were incubated for 48 h. Invasion assays were done with chambers uniformly coated with Matrigel. The cells on the upper surface of the insert were removed, and the insert was fixed in 4% paraformaldehyde (4% PFA) for 15 min, followed by staining with 0.1% crystal violet for 5 min. The stained insert was washed in PBS and observed under a microscope. The average number of stained cells in five fields was used to measure cell migratory ability.

Western blot

Total protein was extracted from cells with radioimmunoprecipitation assay (RIPA) lysis buffer, followed by centrifugation for 15 minutes at 12,000 rpm. The concentration of total protein was quantified using a BCA protein assay kit (Beyotime). Total protein (30 μg) was separated in a 6%, 8%, or 10% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, Burlington, MA, USA). After blocking in 5% non-fat milk for 2 h at room temperature, the membrane was incubated with either anti-YWHAG (Atlas Antibodies, Voltavägen, Sweden; Cat No. HPA026918) or anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat No. sc-47778) primary antibody. The membrane was then incubated with goat anti-rabbit/anti-mouse secondary antibody. Signals were visualized using ECL Substrates (Millipore). The western blotting results were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter assays

Relative reporter gene activity was analyzed in HEK-293T cells cotransfected with psiCHECK2-LYPLAL1-2 (WT or Mut) and miR-NC or miR-217 mimics. After transfection for 48 h, luciferase activity was measured according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) and Prism version 5.0 software (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Unpaired *t*-test was performed for comparison between two groups, and one-way analysis of variance (ANOVA) followed by Bonferroni multiple-comparison test was used to detect differences among more than two groups. Spearman correlation analysis was performed to analyze the statistical dependence between LYPLAL1-2 and miR-217 levels. The differentially expressed LYPLAL1-2 and miR-217 levels between glioma tumor samples and corresponding adjacent noncancerous tissue were compared using a paired *t*-test ($P<0.05$ was considered statistically significant). Cell migration and invasion data were analyzed by ANOVA followed by Bonferroni multiple-comparison test. The luciferase activities of WT or Mut psiCHECK2-LYPLAL1-2 were compared using an unpaired *t*-test ($P<0.05$ was considered statistically significant).

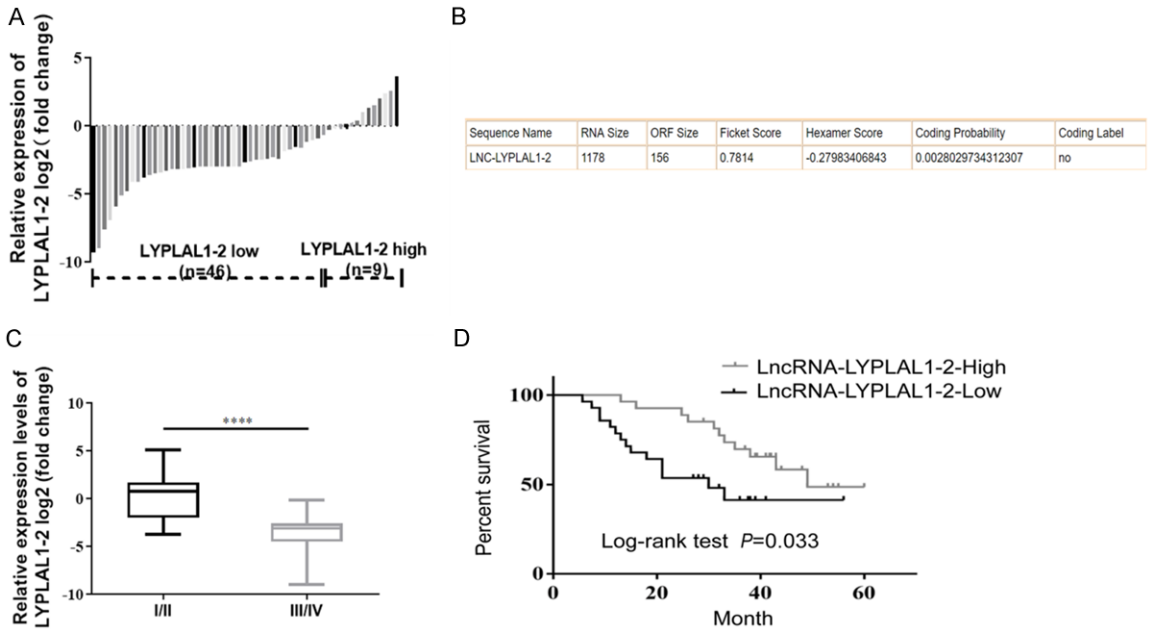

Figure 1. LYPLAL1-2 is significantly downregulated in glioma and correlates with glioma progression. A. qRT-PCR analysis of LYPLAL1-2 expression in glioma and corresponding adjacent noncancerous tissues (n=55). B. The coding capacity of LYPLAL1-2 was determined by online tool Coding-Potential Assessment Tool (CPAT). C. Relative expression level of LYPLAL1-2 in glioma patients with early stage (I-II) and advanced stage (III-IV) disease. D. Kaplan-Meier analysis of overall survival (OS) of all glioma patients stratified by LYPLAL1-2 level. Data are presented as mean ± standard deviation (SD) of three independent experiments. ****P<0.0001.

Results

LncRNA-LYPLAL1-2 is expressed at a low level in glioma tumors and negatively correlates with glioma progression

The level of lncRNA-LYPLAL1-2 in glioma tumor samples and corresponding adjacent noncancerous tissue from 55 patients was determined by qRT-PCR. Results showed that the level of lncRNA-LYPLAL1-2 is low in 83% of 55 patients, whereas only 17% of patients have high expression levels of lncRNA-LYPLAL1-2, compared to that in corresponding adjacent noncancerous tissues (**Figure 1A**). The coding potential of LYPLAL1-2 was determined by the online tool Coding-Potential Assessment Tool (CPAT), which further confirms that LYPLAL1-2 is a long noncoding RNA (**Figure 1B**). Expression of lncRNA-LYPLAL1-2 in patients with early and advanced stage glioma tumors was compared and found to be significantly different ($P<0.0001$). LncRNA-LYPLAL1-2 was found to be highly expressed in early stage (I-II) glioma compared to advanced stage (III-IV) glioma ($P<0.0001$, **Figure 1C**). In our study, we found a clear correlation of lncRNA-LYPLAL1-2 level with tumor size and TNM stage in patients with glioma. However, lncRNA-LYPLAL1-2 level was not significantly correlated with gender, histologic grade, tumor location, or isocitrate dehydrogenase (IDH) mutation status in those patients (**Table 2**). The results of Kaplan-Meier curve analysis according to lncRNA-LYPLAL1-2 level showed that glioma patients with low lncRNA-LYPLAL1-2 level had significantly worse overall survival (OS) than those with high lncRNA-LYPLAL1-2 level (**Figure 1D**). In addition, multivariate Cox regression analysis revealed that lncRNA-LYPLAL1-2 level and gender are independent prognostic factors for OS in glioma patients (**Table 3**). Poor prognosis for patients with low levels of lncRNA-LYPLAL1-2 indicates that lncRNA-LYPLAL1-2 plays an important role in the progression and treatment of glioma patients.

LncRNA-LYPLAL1-2 is expressed at a low level in glioma cell lines and regulates migration and invasive ability of glioma cells

LncRNA-LYPLAL1-2 RNA levels in U251 and U87 glioma cells and astrocytes were analyzed with qRT-PCR, which showed that the level of

Table 2. Relationship between lncRNA-LYPLAL1-2 and clinicopathological characteristics of glioma patients

Characteristic variables	All study patients N=55	LncRNA-LYPLAL1-2		X^2	*P*
		Low (N)	High (N)		
Age (years)				1.643	0.269
<50	21	13	8		
≥50	34	15	19		
Gender				1.735	0.227
Male	41	23	18		
Female	14	5	9		
Histologic grade				0.210	0.781
I-II	20	11	9		
III-IV	35	17	18		
T stage				6.557	[a]0.015
I-II	25	8	17		
III-IV	30	20	10		
Tumor location				0.439	0.582
Frontal	35	19	16		
Temporal	20	9	11		
Tumor size (cm)				5.263	[a]0.031
<4.5	24	8	16		
≥4.5	31	20	11		
IDH mutation status				0.881	0.412
mutated	21	9	12		
wild type	34	19	15		

[a]Statistically significant. IDH, isocitrate dehydrogenase.

Table 3. Multivariate analysis using Cox regression analysis demonstrates that lncRNA-LYPLAL1-2 is independent prognostic factor for glioma patients

Characteristic variables	Overall survival	
	P	HR (95% CI)
Age	0.286	1.801 (0.611-5.310)
Gender	[a]0.021	2.997 (1.176-7.638)
Tumor grade	0.805	0.888 (0.345-2.283)
T stage	0.624	0.787 (0.302-2.050)
IDH mutation status	0.770	1.161 (0.428-3.146)
Tumor size	0.943	0.963 (0.346-2.681)
Tumor location	0.396	1.489 (0.594-3.732)
LncRNA-LYPLAL1-2	[a]0.022	3.551(1.205-10.467)

[a]Statistically significant. HR, hazard ratio; CI, confidence interval.

lncRNA-LYPLAL1-2 is significantly lower in U251 and U87 cellscompared to astrocytes (*P*<0.001, **Figure 2A**). To investigate the role of lncRNA-LYPLAL1-2 in the invasion of glioma, both glioma cell lines were successfully transfected with OEC or OE-LYPLAL1-2; this was confirmed by qRT-PCR 72 h after transfection (**Figure 2B**). The transwell assays were then used to identify the migration and invasive capacity of U87 and U257 cells transfected with OEC or OE-LYPLAL1-2. Few U251 cells transfected with OE-LYPLAL1-2 passed through the membrane and appeared on the bottom surface of the insert compared to U87 cells transfected with OEC. The U87 cells transfected with OE-LYPLAL1-2 also shower weaker invasive capacity than U87 cells transfected with OEC (*P*<0.001, **Figure 2C** and **2D**).

LncRNA-LYPLAL1-2 directly targets miR-217 in glioma cells

The binding site of miR-217 was reviewed and compared with that of WT or Mut LYPLAL1-2 in **Figure 3A**. The results indicate miR-217 has the ability to bind strongly to lncRNA-LYPLAL1-2. MiR-217 expression in glioma tumors and corresponding adjacent noncancerous tissue of 55 glioma patients was analyzed by qRT-PCR. MiR-217 was found to be highly expressed in 43 (78%) of the 55 glioma tumors (**Figure 3B**). The association between LYPLAL1-2 and miR-217 levels was examined by Spearman correlation analysis in 55 paired glioma tissues. The findings indicate that LYPLAL1-2 and miR-217 levels were highly negatively associated with each other (*P*<0.05, **Figure 3C**). Moreover, the abundance of miR-217 in glioma patients with early stage (I-II) and advanced stage (III-IV) disease was compared by qRT-PCR and found to be significantly higher in advanced

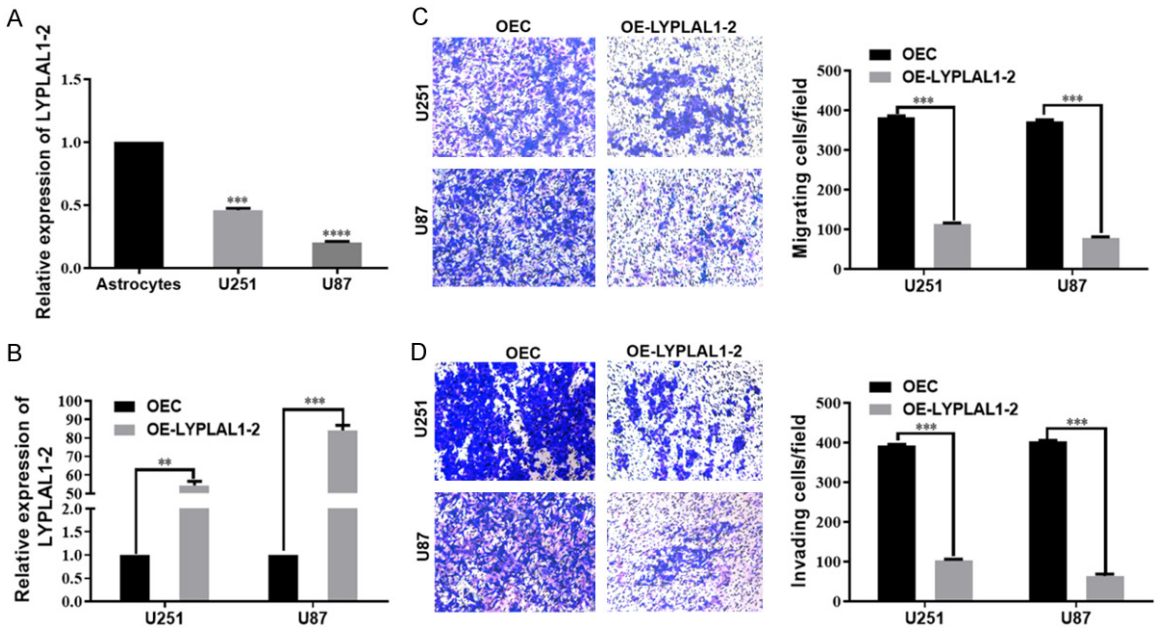

Figure 2. LYPLAL1-2 is significantly downregulated in glioma cells and suppresses glioma cells invasion and tumor metastasis *in vitro*. A. qRT-PCR analysis of LYPLAL1-2 expression in glioma cells and astrocytes. B. qRT-PCR analysis of LYPLAL1-2 expression in control and LYPLAL1-2-transfected glioma cells. C and D. Transwell assays were used to determine the cell migratory and invasive capacity of LYPLAL1-2-transfected glioma cells. Data are presented as mean ± SD of three independent experiments. **P<0.0001, ***P<0.0001, ****P<0.0001.

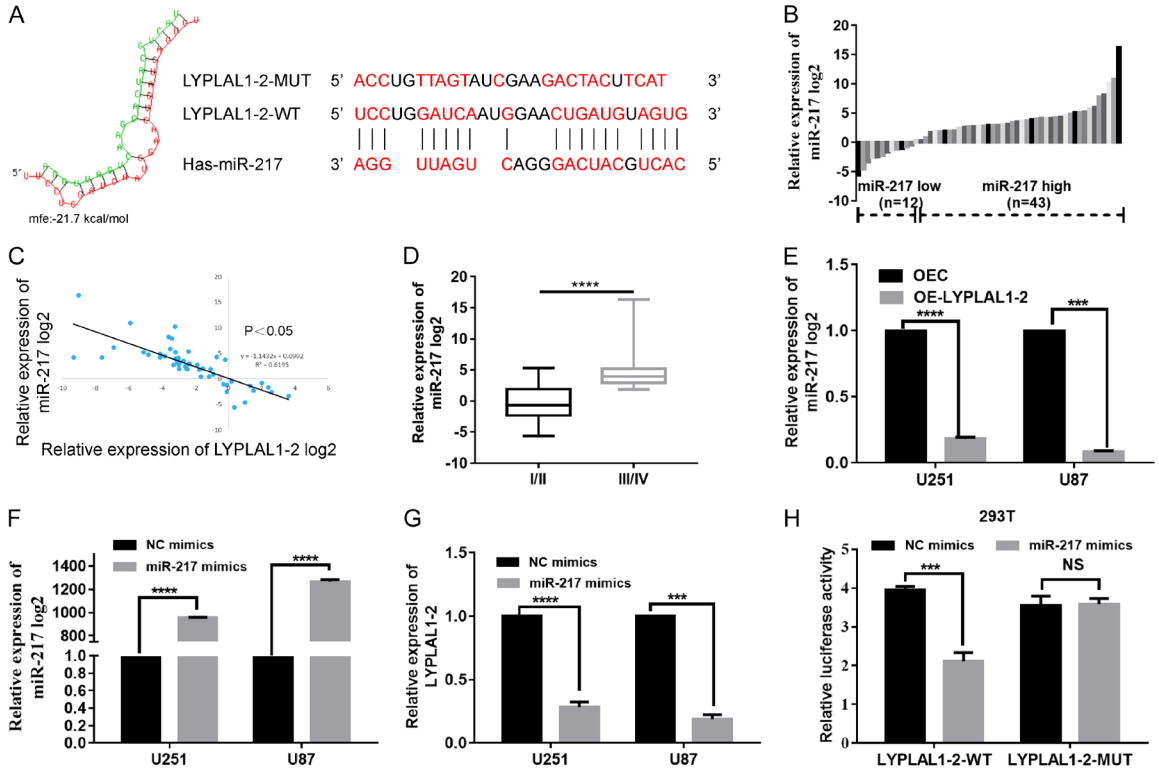

Figure 3. LYPLAL1-2 interacts with miR-217. A. Schematic view of miR-217 binding site in WT or Mut LYPLAL1-2. B. MiR-217 expression was analyzed by qRT-PCR in glioma and corresponding adjacent noncancerous tissues (n=55). C. The association between LYPLAL1-2 and miR-217 expression levels was identified with Spearman correlation analysis in 55 paired glioma tissues. D. Relative expression level of miR-217 in glioma patients with early stage

(I-II) and advanced stage (III-IV) disease. Data are presented as mean ± SD of three independent experiments. E. qRT-PCR assay was performed to detect the expression level of miR-217 in LYPLAL1-2-transfected glioma cells. F. qRT-PCR assay was performed to detect the expression level of miR-217 in glioma cells transfected with miR-217 mimics. G. qRT-PCR assay was performed to detect the expression level of LYPLAL1-2 in miR-217-transfected glioma cells. H. Relative reporter gene activity of the psiCHECK2-LYPLAL1-2 (WT or Mut) in HEK-293T cells co-transfected with miR-NC or miR-217 mimics. Data are presented as mean ± SD of three independent experiments. **P<0.01, ***P<0.001, ****P<0.0001.

stage than in early stage (*P*<0.0001, **Figure 3D**). The level of miR-217 in glioma cells (U251 and U87) transfected with OEC or OE-LYPLAL1-2 were analyzed by qRT-PCR. Results indicate that miR-217 was expressed at a lower level in U251 and U87 glioma cells transfected with OE-LYPLAL1-2 than in the same cell types transfected with OEC (U251, *P*<0.0001; U87, *P*<0.001; **Figure 3E**). Both glioma cell lines were successfully transfected with NC mimics or miR-217 mimics, as confirmed by qRT-PCR. miR-217 was found to be expressed at a high level in U251 (*P*<0.0001) and U87 cells (*P*<0.0001) transfected with miR-217 mimics (**Figure 3F**). After transfection of U251 and U87 cells with NC mimics or miR-217 mimics, the level of LYPLAL1-2 in the cells was analyzed by qRT-PCR and indicated that LYPLAL1-2 level was lower in both types of glioma cells transfected with miR-217 mimics than in cells transfected with NC mimics (U251, *P*<0.001; U87, *P*<0.001, **Figure 3G**). The luciferase activity of HEK-293T cells was found to be significantly reduced when cotransfected with the psi-CHECK2-LYPLAL1-2 (WT) and miR-217 mimics (*P*<0.001, **Figure 3H**).

LncRNA-LYPLAL1-2 overexpression suppresses glioma cell migration and invasion via sponging to miR-217

The function of miR-217 in glioma was further explored. Transwell assays were used to determine the migration and invasive capacity of U251 and U87 glioma cells cotransfected with LYPLAL1-2 + NC mimics, LYPLAL1-2 + miR-217 mimics, or OEC+ NC mimics. Glioma cells transfected with LYPLAL1-2 + NC mimics presented weaker invasive capacity compared to the OEC + NC mimics group (P<0.001, **Figure 4A**). Similar results were observed for invasion (P<0.001, **Figure 4B**). These results indicated that LYPLAL1-2 acts as a molecular sponge and inhibits glioma cell migration and invasion via downregulation of miR-217.

YWHAG is the direct target gene of miR-217 and is downregulated by lncRNA-LYPLAL1-2

To confirm the mechanism underlying the activity of the LYPLAL1-2/miR-217 axis, potential targets of miR-217 were predicted with TargetScan. According to the prediction, miR-217 can directly bind to the YWHAG 3'UTR (**Figure 5A**). Dual-luciferase reporter assay showed that luciferase activity was markedly repressed in HEK-293T cells co-transfected with YWHAG-3'UTR WT and miR-217 mimics (*P*<0.01, **Figure 5B**). These data indicated that miR-217 directly binds to the 3'UTR of YWHAG.

Subsequently, to determine the interaction between LYPLAL1-2, miR-217, and YWHAG, U251 and U87 cells were co-transfected with LYPLAL1-2 and miR-217 mimic, and the expression level of YWHAG was analyzed by qRT-PCR and western blotting. As shown in **Figure 5C** and **5D**, both in mRNA and protein level, overexpression of miR-217 effectively reversed the production of YWHAG induced by LYPLAL1-2 overexpression. Together with these observations, it can be verified that YWHAG was indirectly regulated by LYPLAL1-2 and involved in glioma cells migration and invasion.

YWHAG is markedly downregulated in glioma tumors and positively correlated with the expression of LYPLAL1-2 and miR-217

qRT-PCR analysis was performed to illustrate the level of YWHAG mRNA in glioma tumors and corresponding adjacent noncancerous tissues of 55 glioma patients. YWHAG level was observed to be low in 49 (89%) glioma tumors of the 55 glioma tumors compared to adjacent noncancerous tissue (**Figure 6A**). The association between YWHAG and glioma tumor stage was investigated and showed that YWHAG is expressed at a high level in early stage (I-II) glioma tumors, compared to late stage (III-IV) glioma tumor (*P*<0.0001, **Figure 6B**). The relationship between LYPLAL1-2 or miR-217 level and YWHAG level was studied with Spearman cor-

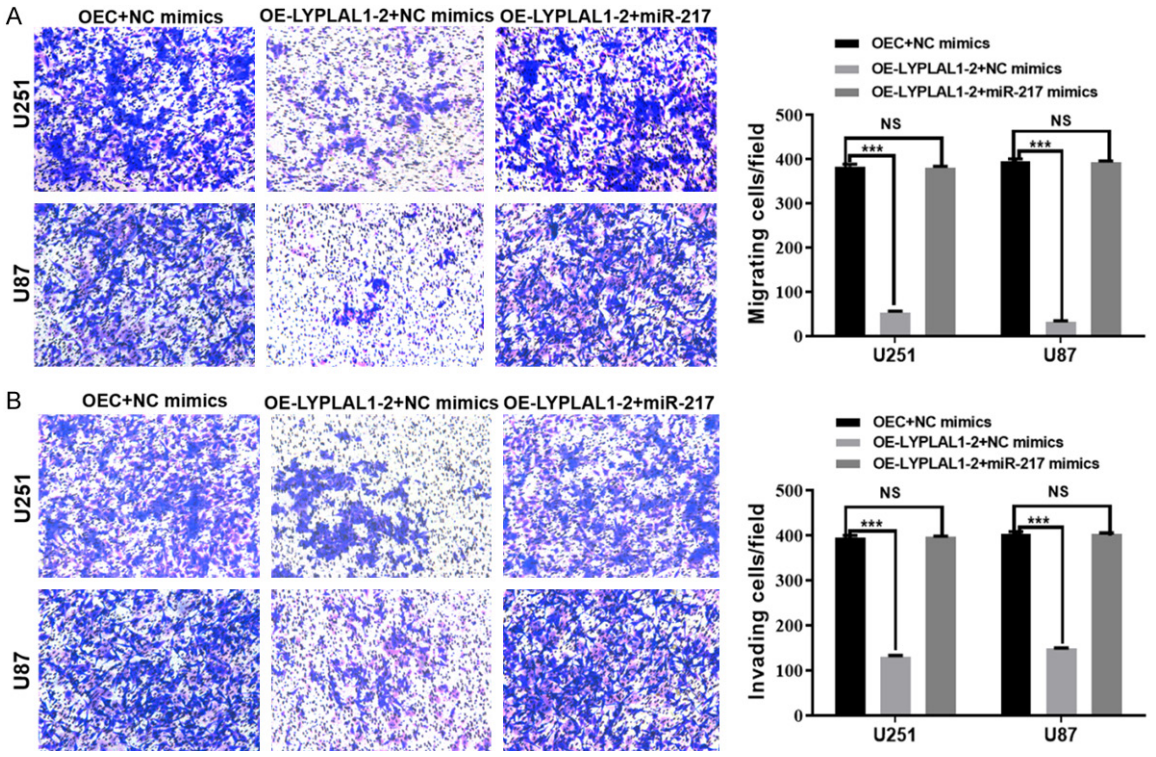

Figure 4. LncRNA-LYPLAL1-2 regulates glioma cell migration and invasion via sponging miR-217. Transwell assays were used to determine glioma cell migration (A) and invasion (B) after co-transfection with LYPLAL1-2 and miR-217 mimics. Data are presented as mean ± SD of three independent experiments. ***P<0.001.

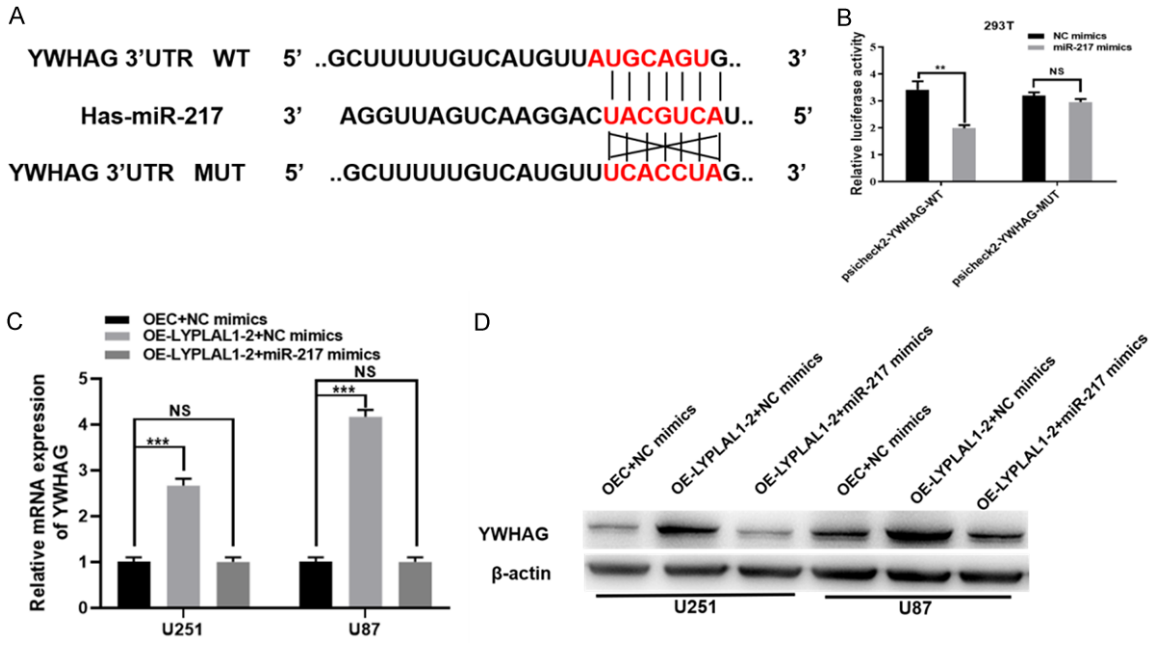

Figure 5. LYPLAL1-2 regulates YWHAG expression by sponging miR-217. (A) Schematic diagram of the potential binding site of YWHAG within miR-217. (B) Dual-luciferase reporter assay in HEK-293T cells treated with wild-type or mutant YWHAG and miR-217 mimics or NC. (C) The mRNA and (D) protein levels of YWHAG in glioma cells co-transfected with LYPLAL1-2 and miR-217 mimics or their negative control. Data are presented as mean ± SD of three independent experiments. ***P<0.001.

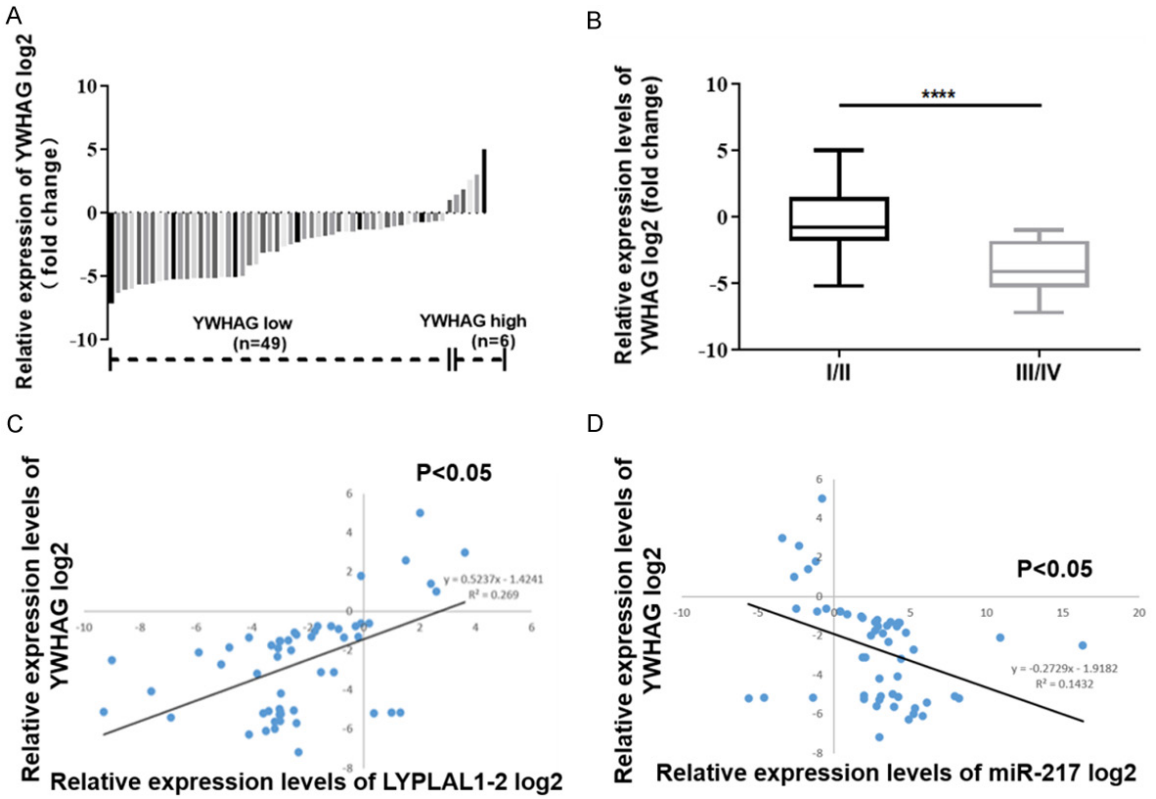

Figure 6. The association between LYPLAL1-2, miR-217, and YWHAG levels in glioma tumors. A. YWHAG level was analyzed by qRT-PCR in glioma and corresponding adjacent noncancerous tissue (n=55). B. Relative expression level of YWHAG in glioma patients with early stage (I-II) and advanced stage (III-IV) disease. C. The association between LYPLAL1-2 and YWHAG levels was identified with Spearman correlation analysis in 55 paired glioma tissues. D. The association between miR-217 and YWHAG expression levels was identified with Spearman correlation analysis in 55 paired glioma tissues. Data are presented as mean ± SD of three independent experiments. ****P<0.0001.

relation analysis in 55 paired glioma tissues. Results of the analysis indicate that LYPLAL1-2 and YWHAG levels are strongly positively associated with each other (P<0.05, **Figure 6C**), whereas miR-217 and YWHAG levels are highly negatively associated with each other (P<0.05, **Figure 6D**).

LncRNA-LYPLAL1-2 overexpression inhibits glioma tumor growth and metastasis in vivo

To verify the functions and underlying mechanisms of lncRNA-LYPLAL1-2 in glioma metastasis, U87 glioma cells were injected with OEC or OE-LYPLAL1-2 into the subcutaneous of mice. Thirty days after injection, mice were sacrificed and lungs and livers collected for H&E staining. As shown in **Figure 7A** and **7B**, the glioma tumors derived from OE-LYPLAL1-2 mice were smaller than those derived from the OEC group, and fewer metastatic nodules were formed in OE-LYPLAL1-2 mice than in the OEC group, both in liver and lung. These results reveal that glioma mice treated with OE-LYPLAL1-2 present weak malignant morphology compared to those injected with OEC (**Figure 7C**). In addition, the expression levels of miR-217 and YWHAG were regulated by LYPLAL1-2 *in vivo*. Overexpression of LYPLAL1-2 reduced miR-217 level and increased YWHAG level (**Figure 7D** and **7E**). Results of experiments showed that OE-LYPLAL1-2 is able to suppress tumor growth and metastasis *in vivo*.

Discussion

Glioma has been recognized as the most common and aggressive malignant CNS tumor and is associated with a high mortality rate [22, 23]. The overall survival of glioma patients still remains low, despite multiple therapeutic approaches [24, 25]. Therefore, it is urgent to explore the underlying causes of glioma to aid in its treatment.

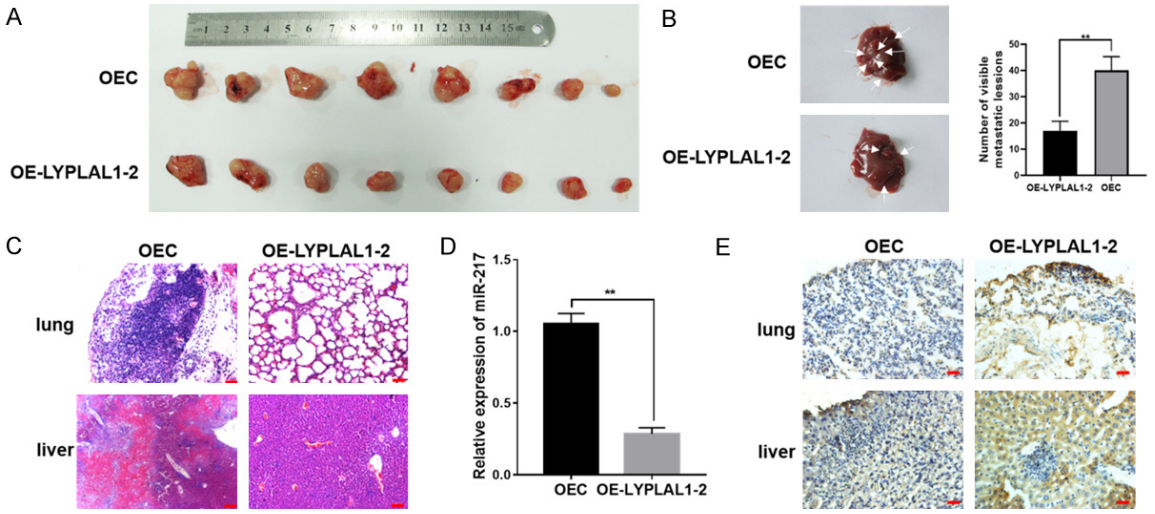

Figure 7. LYPLAL1-2 suppresses glioma tumor growth and metastasis *in vivo*. A. U87 glioma cells were injected with OEC or OE-LYPLAL1-2 into the subcutaneous of mice. Tumors were excised 30 days after injection. Xenografts show modest growth inhibition in nude mice harboring OE-LYPLAL1-2 versus OEC (n=8/group). ***P*<0.01. B. Representative images of livers (left) and quantitative data (right) of mice harboring OE-LYPLAL1-2 xenografts indicate number of metastatic colonies; **P<0.01 versus control. C. Representative images of metastatic deposits in H&E-stained lungs and livers of nude mice harboring OE-LYPLAL1-2 or OEC xenografts. D. miR-217 level was detected by qRT-PCR. E. YWHAG level was analyzed by immunohistochemistry. Scale bars = 50 μm. Data are presented as mean ± SD of three independent experiments. ***P*<0.01.

Increasing studies confirm that lncRNAs play pivotal roles in various malignancies [26, 27]. In the study published by Yi Chen et al. [16], lncRNA-LYPLAL1-2 level is reported to be low in recurrent gliomas compared with primary gliomas. In our study, lncRNA-LYPLAL1-2 expression was also found to be significantly lower in glioma tumors than in the corresponding adjacent healthy tissue and in glioma cell lines compared to astrocytes. All of the above findings indicate lncRNA-LYPLAL1-2 was negatively associated with glioma. Moreover, lncRNA-LYPLAL1-2 was found to be abundant in early stage glioma, indicating that it was negatively correlated with glioma progression. All of the above results suggest that lncRNA-LYPLAL1-2 plays a vital role in the progression of glioma.

To determine the exact role of lncRNA-LYPLAL1-2 in glioma, the migration and invasive capacity of glioma cell lines U251 and U87 overexpressing lncRNA-LYPLAL1-2 were quantified and found to be weakened. In addition, the metastasis of glioma tumor cells in mice is suppressed when serum level of lncRNA-LYPLAL1-2 is high. The previous study also found lncRNA-LYPLAL1-2 was downregulated in pancreatic ductal adenocarcinoma compared to the adjacent noncancerous tissue [15].

To elucidate the possible mechanism by which lncRNA-LYPLAL1-2 regulates the migration and invasion of glioma cells, its target miRNAs were identified by luciferase reporter assay in our study, showing that miR-217 is the target of lncRNA-LYPLAL1-2. MiR-217 expression level is high both in glioma tumors compared to corresponding adjacent healthy tissue and in U251 and U87 glioma cells compared to astrocytes. Moreover, the miR-217 level was found to be positively associated with the progression of glioma in our study, which is to some degree consistent with Wang H et al' s study [19]. That group confirmed that miR-217 promotes the proliferation and invasion of glioblastoma. These data imply that lncRNA-LYPLAL1-2 targets and negatively regulates miR-217 in glioma cells. Moreover, we confirmed that miR-217 directly targets the 3'UTR of YWHAG and suppresses YWHAG expression in glioma cells, which is also consistent with the results from Wang H et al's study [19]. Thus, we conclude that lncRNA-LYPLAL1-2 modulates glioma metastasis via regulation of the miR-217/YWHAG axis.

These results might provide novel targets for glioma therapy. Further research is still needed to address and further confirm our findings.

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Disclosure of conflict of interest

None.

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