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APLNR stimulates the development of glioma *via* the NFAT5/AKT feedback loop

Z.-P. ZHANG, X.-Q. SUN, S.-J. YUAN

Department of Brain Center, Sunshine Integration Hospital Shandong Chine, Weifang, China

Abstract. – OBJECTIVE: To detect the role of APLNR in influencing the proliferative ability and development of glioma.

PATIENTS AND METHODS: APLNR levels in 42 matched glioma tissues and adjacent normal brain tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between APLNR level and clinical features of glioma patients was assessed. Regulatory effects of APLNR on glioma cell functions were evaluated by cell counting kit-8 (CCK-8), colony formation, and 5-Ethynyl-2'-deoxyuridine (EdU) assay, respectively. At last, the involvement of NFAT5 in APLNR-regulated glioma cell phenotypes was examined.

RESULTS: APLNR was upregulated in glioma tissues than the adjacent ones. Glioma patients expressing higher level of APLNR had more advanced stage and worse prognosis. Knockdown of APLNR inhibited proliferative ability of glioma. NFAT5 level was negatively regulated by APLNR. Notably, NFAT5 could partially abolish the regulatory effect of APLNR on glioma cell phenotypes.

CONCLUSIONS: APLNR level is closely linked to tumor grading and prognosis of glioma patients. It stimulates proliferative ability in glioma cells by targeting NFAT5.

Key Words:
APLNR, NFAT5, Glioma, Proliferation.

Introduction

Glioma is a common cranial malignancy, accounting for 60% of central nervous system tumors. It is one of the five most incurable tumors throughout the world[1-3]. The prognosis of glioma is extremely poor due to its refractoriness, high rates of local infiltration, and recurrences[4,5]. Although great strides have been made on comprehensive therapies for glioma, including surgery, chemotherapy, radiotherapy, and biological immunotherapy, the 5-year survival of glioma is only about 5%[6,7]. Low detective rate of glioma in the early stage is the major reason for it poor prognosis. Generally speaking, infiltration and metastasis of glioma occur at the initial diagnosis[8,9]. Biopsy is the gold standard for diagnosing glioma. Nevertheless, biopsy results in big trauma due to the deep anatomic location[10,11]. Therefore, it is urgent to search for an effective and specific biomarker for glioma[12,13].

Tumorigenesis is a complicated process involving external and internal factors[14,15]. Tumor cell proliferation is a key event during the malignant development of tumors[16]. Apelin receptor (APLNR), also known as APJ or AGTRL1, exerts a critical role in stimulating proliferative ability and angiogenesis. APLNR is a tumor-associated gene[17,18]. In addition, NFAT5 could specifically bind APLNR through bioinformatics analysis. NFAT5 is a newly discovered member in the NFAT family, which has 40% homology in the Rel homology region to NFAT1-4. However, NFAT5 is the only member of the NFAT family that is not regulated by calcium ions since sequences binding calcineurin at the amino terminus are lacking[19-21]. So far, the interaction between APLNR and NFAT5 in the development of glioma is rarely reported.

In this paper, we first detected differential expressions of APLNR in glioma tissues and normal brain tissues. Subsequently, APLNR level in several commonly used glioma cell lines was detected. Glioma cells with a relatively high abundance of APLNR were used to explore regulatory effects of APLNR and NFAT5 on glioma. Our findings may provide new ideas for clinical diagnosis and treatment of glioma.

Patients and Methods

Patients and Glioma Samples

A total of 42 glioma tissues and matched normal brain tissues surrounding glioma were collected from the glioma treatment center and were independently diagnosed and graded by two pathologists. Tumor grading was assessed based on the standard criteria proposed by UICC. Patients and their families in this study have been fully

Corresponding Author: Shaoji Yuan, MD; e-mail: zhao-peng-zhang@163.com

informed. This investigation was approved by the Ethics Committee of Sunshine Integration Hospital Shandong Chine.

Cell Culture

The human glioma cell lines (U251, U87, T98-G, A172) and human brain normal glial cells (HEB) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Sh-NC and sh-APLNR were provided by GenePharma (Shanghai, China). Cells were inoculated in a 6-well plate and cultured to 30-40% confluence. Cells were transfected with corresponding plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in a 6-well plate with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Afterwards, visible colonies were washed in phosphate-buffered saline (PBS), fixed in methanol for 20 min, and dyed in 0.1% crystal violet for 20 min. Finally, colonies were captured and calculated.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

The cells were incubated with 50 μm EdU solution (RiboBio, Nanjing, China) for 2 h, followed by staining with AdoLo and 4',6-diamidino-2-phenylindole (DAPI). Finally, EdU-positive cells were captured under a fluorescence microscope.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA in cells or tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was applied to qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were listed as follows: APLNR: forward: 5'-TCTTGGCTCTTCCCTCTTTTCA-3', reverse: 5'-GTGCTGGAATCCACTGGAGAA-3'; NFAT5: forward: 5'-GAAGTGGACATTGAAGGCACT-3', reverse: 5'-CTGGCTTCGACATCAGCATT-3'; GAPDH: forward: 5'-TGCACCACCAACTGCTTAGC-3', reverse: 5'-GGCATGCACTGTGGTCATGAG-3'.

Western Blot

Total protein was extracted from cells or tissues. The obtained protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked in 5% skim milk for 1 h. The specific primary antibody was used to incubate with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 h at room temperature. After Tris-Buffered Saline and Tween-20 (TBST) washing for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables were analyzed by the *t*-test, and categorical variables were analyzed by χ^2-test or Fisher's exact test. Kaplan-Meier curves were depicted for survival analysis. $p<0.05$ was considered as statistically significant.

Results

APLNR Was Highly Expressed in Glioma

Compared with adjacent normal brain tissues, APLNR was upregulated in glioma tissues (Figure 1A). Identically, its level was highly expressed in glioma cells (Figure 1B). In particular, A172 and U87 cell lines expressed the highest abun-

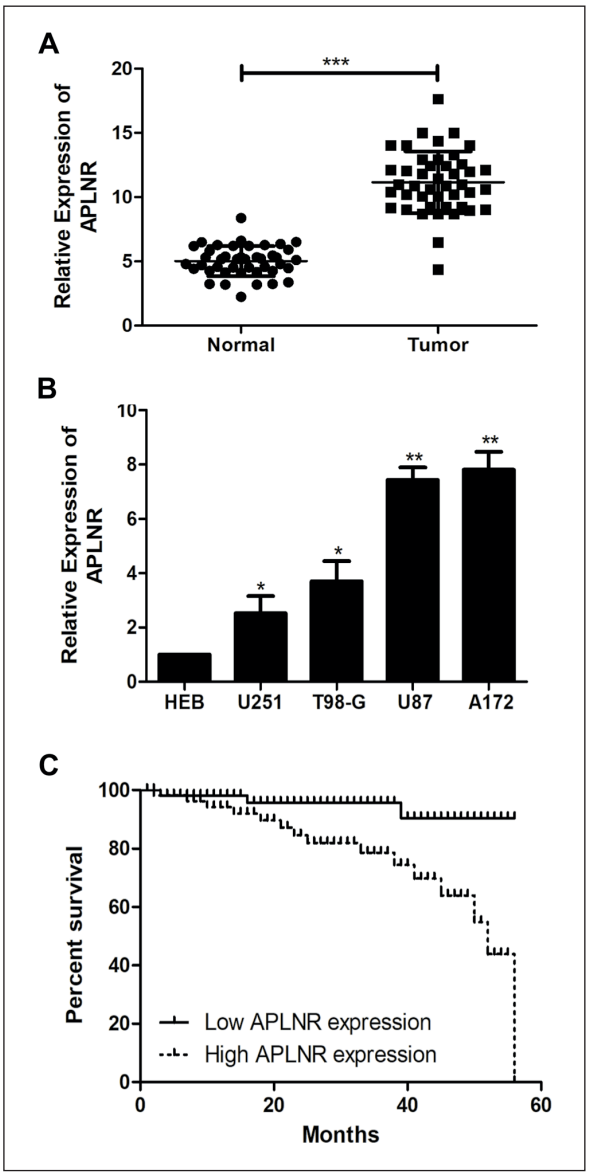

Figure 1. APLNR was highly expressed in glioma. **A**, APLNR levels in glioma tissues and adjacent normal tissues. **B**, APLNR level in glioma cell lines. **C**, Overall survival in glioma patients with high or low level of APLNR. Data were expressed as mean±SD *p<0.05, **p<0.01, ***p<0.001.

dance of APLNR among the four tested glioma cell lines, which were selected for the following experiments.

APLNR Expression Was Correlated with Pathological Stage and Poor Prognosis in Glioma Patients

Clinical data of enrolled glioma patients were collected. It is shown that APLNR level was positively correlated to pathological stage of glioma patients, while it was unrelated to age, sex, lymphatic metastasis, and distant metastasis (Table I). In addition, Kaplan-Meier curves illustrated a poor prognosis in glioma patients expressing high level of APLNR (Figure 1C).

Knockdown of APLNR Inhibited Proliferative Ability in Glioma

Two APLNR shRNAs were conducted in this experiment. Transfection of either sh-APLNR-1 or sh-APLNR-2 could effectively downregulate APLNR in A172 and U87 cells (Figure 2A). The former one was adopted due to its better transfection efficacy. CCK-8 assay showed a remarkable decline in cell viability after knockdown of APLNR in glioma cells (Figure 2B). Similarly, decreased colony number (Figure 2C) and EdU-positive ratio (Figure 2D) after transfection of sh-APLNR-1 both suggested the inhibited proliferative ability in glioma cells.

NFAT5 Was Lowly Expressed in Glioma

A potential interaction between APLNR and NFAT5 was predicted by bioinformatics analysis (data not shown). Moreover, decreased Luciferase activity after co-transfection of pcDNA-NFAT5 and APLNR-WT further showed their binding relationship (Figure 3A). NFAT5 was lowly expressed in glioma tissues (Figure 3B) and cell lines (Figure 3C). As expected, a negative correlation was discovered between expression levels of APLNR and NFAT5 in glioma tissues (Figure 3D). Knockdown of APLNR could upregulate protein level of NFAT5 and downregulate protein level of AKT in glioma cells (Figure 3E).

APLNR/NFAT5 Axis Regulated Glioma Cell Behaviors

To uncover the involvement of NFAT5 in the development of glioma, co-transfection of si-NFAT5 and sh-APLNR-1 was performed. Both qRT-PCR and Western blot data revealed higher level of APLNR and lower level of NFAT5 in glioma cells co-transfected with si-NFAT5 and sh-APLNR-1 than those with solely knockdown of APLNR (Figure 4A, 4B). Decreased viability in glioma cells with APLNR knockdown was partially reversed by NFAT5 knockdown (Figure 4C). In addition, knockdown of NFAT5 could abolish the decreased colony number (Figure 4D) and EdU-positive ratio (Figure 4E) in glioma cells with APLNR knockdown. Hence, NFAT5 was involved in the proliferation of glioma cells regulated by APLNR.

Table I. Association of APLNR expression with clinicopathologic characteristics of glioma.

Parameters	Number of cases	APLNR expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.361
<60	22	13	9	
≥60	20	9	11	
Gender				0.361
Male	20	9	11	
Female	22	13	9	
T stage				0.013
T1-T2	27	18	9	
T3-T4	15	4	11	
Lymph node metastasis				0.808
No	26	14	12	
Yes	16	8	8	
Distance metastasis				0.126
No	28	17	11	
Yes	14	5	9	

Discussion

Glioma derives from the central nerves system, which is featured by high invasiveness, easy recurrence, and high mortality[3-6]. The prognosis of glioma is extremely poor even after active treatment[7-10]. It is necessary to uncover the pathogenesis of glioma, thus improving life quality and survival of affected patients[11-13].

Tumor development involves dysfunctional cells and pathways[14,15]. Imbalanced oncogenes and tumor suppressor genes are responsible for this process[15]. Currently, epigenetics has been well concerned during tumorigenesis[15,16]. APLNR is reported to be closely linked to malignant proliferation of tumor cells[17,18]. This study aims to detect differential expression of APLNR in glioma samples and biological changes in glioma cells after APLNR intervention, thereby clarifying the role of APLNR in the development of glioma. Here, we collected 42 paired glioma tissues and paracancerous ones. APLNR was upregulated in glioma tissues than the adjacent ones. Glioma patients expressing higher level of APLNR had ad-

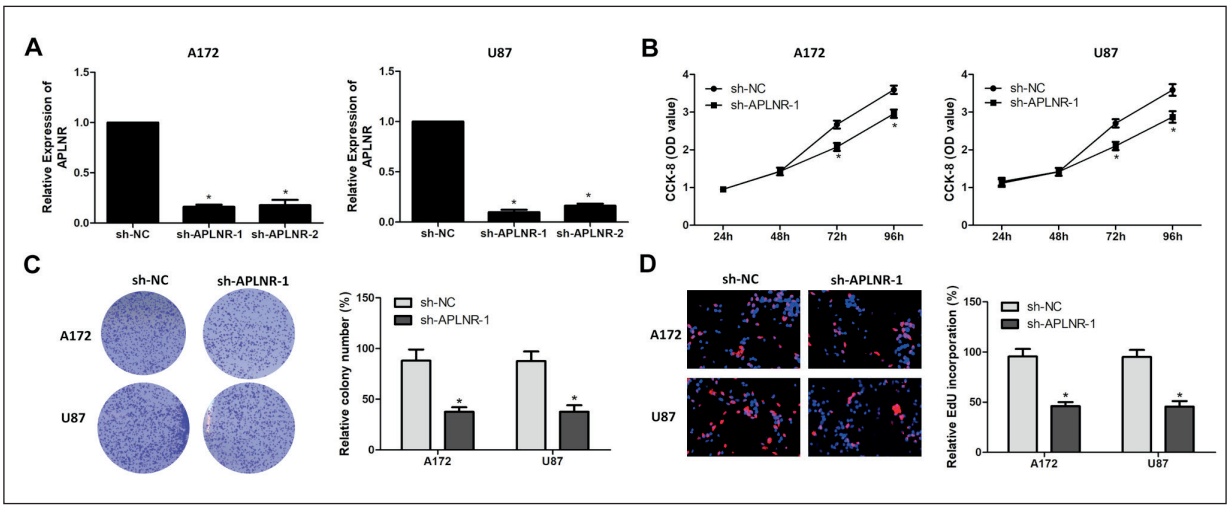

Figure 2. Knockdown of APLNR inhibited proliferative ability in glioma. **A**, Transfection efficacy of sh-APLNR-1 and sh-APLNR-2 in A172 and U87 cells. **B**, Viability in A172 and U87 cells transfected with sh-NC or sh-APLNR-1. **C**, Colony number in A172 and U87 cells transfected with sh-NC or sh-APLNR-1 (magnification 10×). **D**, EdU-positive ratio in A172 and U87 cells transfected with sh-NC or sh-APLNR-1 (magnification 40×). Data were expressed as mean±SD *p<0.05.

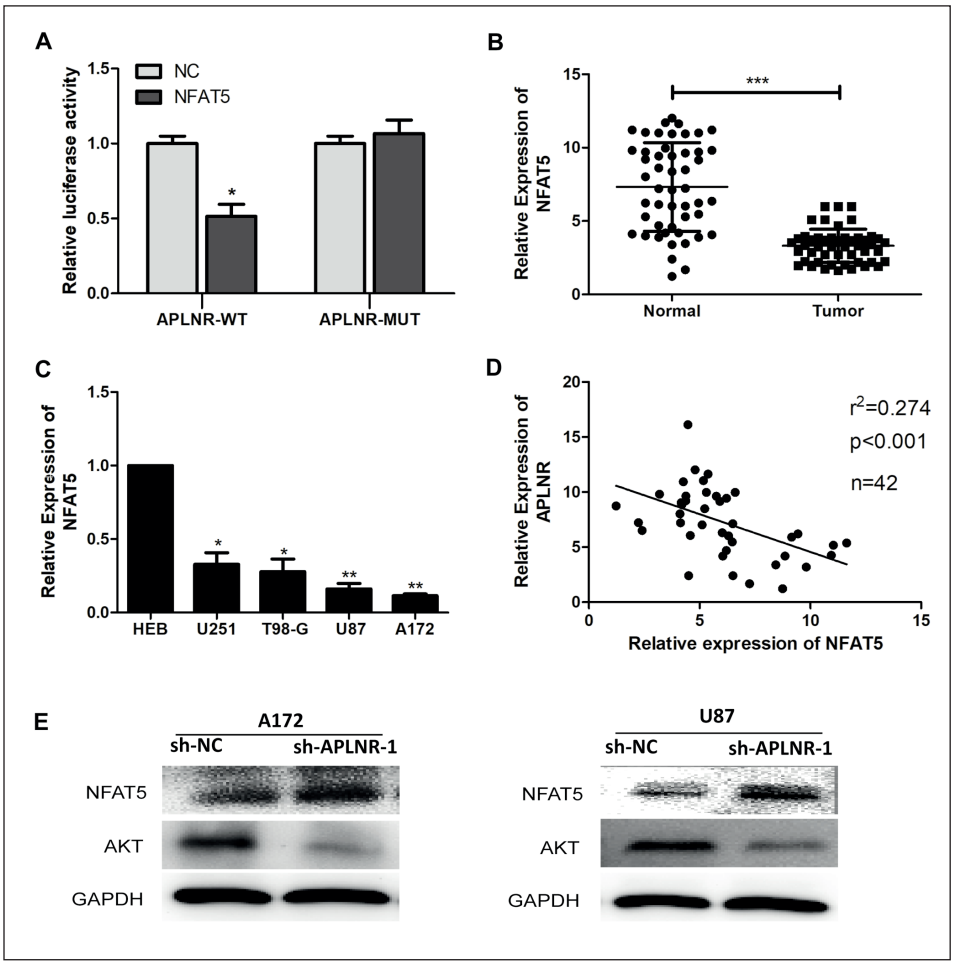

Figure 3. NFAT5 was lowly expressed in glioma. **A**, Luciferase activity after co-transfection of NC/pcDNA-NFAT5 and APLNR-WT/APLNR-MUT. **B**, NFAT5 levels in glioma tissues and adjacent normal tissues. **C**, NFAT5 level in glioma cell lines. **D**, A negative correlation between expression levels of APLNR and NFAT5 in glioma tissues. **E**, Protein levels of NFAT5 and AKT in A172 and U87 cells transfected with sh-NC or sh-APLNR-1. Data were expressed as mean±SD *p<0.05, **p<0.01, ***p<0.001.

vanced stage and worse prognosis. We suggested that APLNR was a carcinogenic gene aggravating the development of glioma.

Tumorigenesis is a complicated multi-stage process involving both external and internal stimuli[14-16]. To explore the *in vitro* influences of APLNR on glioma cell phenotypes, APLNR knockdown model was constructed by lentivirus transfection. CCK-8, colony formation, and EdU assay all demonstrated that APLNR could stimulate proliferation of glioma cells. APLNR was of significance during the deterioration of glioma, while its molecular mechanisms remained unclear.

Target genes of APLNR were subsequently searched and their interaction in the glioma development was mainly explored. Bioinformatics prediction and Dual-Luciferase reporter assay confirmed that NFAT5 was the target gene of APLNR. NFAT5 was lowly expressed in glioma tissues and cell lines. Its level was negatively regulated by APLNR. Notably, NFAT5 was responsible for APLNR-regulated proliferative ability in glioma cells. Collectively, APLNR/NFAT5 axis was identified to deteriorate the development of glioma.

Conclusions

APLNR level is closely linked to tumor grading and prognosis of glioma patients. It stimulates proliferative ability in glioma cells by targeting NFAT5.

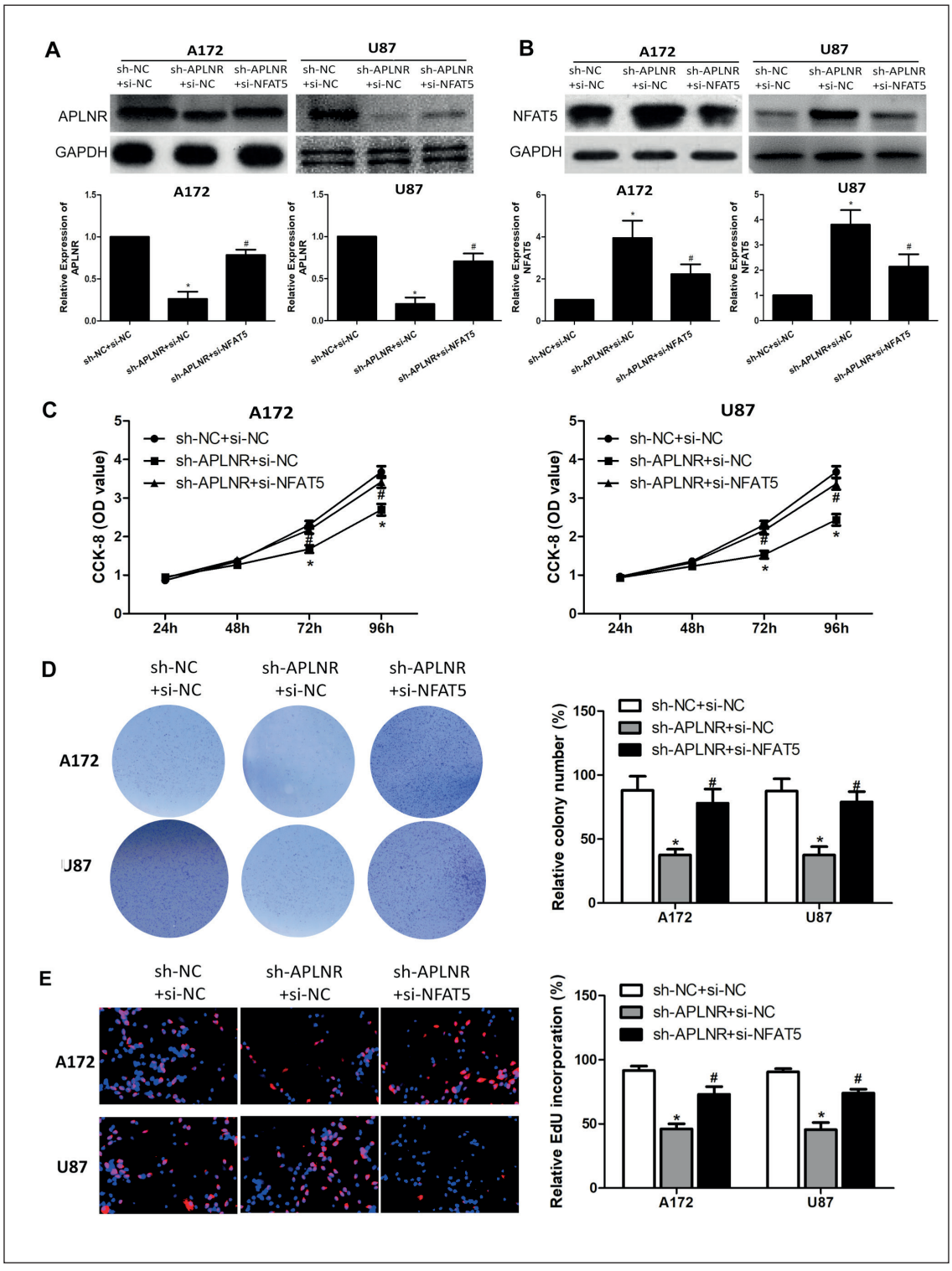

Figure 4. APLNR/NFAT5 axis regulated glioma cell behaviors. **A**, APLNR level in A172 and U87 cells transfected with sh-NC+si-NC, sh-APLNR-1+si-NC or sh-APLNR-1+si-NFAT5. **B**, NFAT5 level in A172 and U87 cells transfected with sh-NC+si-NC, sh-APLNR-1+si-NC or sh-APLNR-1+si-NFAT5. **C**, Viability in A172 and U87 cells transfected with sh-NC+si-NC, sh-APLNR-1+si-NC or sh-APLNR-1+si-NFAT5. **D**, Colony number in A172 and U87 cells transfected with sh-NC+si-NC, sh-APLNR-1+si-NC or sh-APLNR-1+si-NFAT5 (magnification 10×). **E**, EdU-positive ratio in A172 and U87 cells transfected with sh-NC+si-NC, sh-APLNR-1+si-NC or sh-APLNR-1+si-NFAT5 (magnification 40×). Data were expressed as mean±SD *#$p<0.05$.

Conflict of Interests

The authors declare that they have no conflict of interests.

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