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Circular RNA circ_0079593 promotes glioma development through regulating KPNA2 expression by sponging miR-499a-5p

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Abstract. – OBJECTIVE: Glioma is a common aggressive cancer and a major public health problem worldwide, with high incidence, recurrence, and mortality. Circular RNA (circRNA) has been reported to be involved in glioma, but the role of circ_0079593 in glioma is still unclear.

MATERIALS AND MATERIALS: The re[illegible] quantitative polymerase chain reaction [illegible] CR) was performed to quantify the e[illegible]s-sion levels of circ_0079593, miR-499a-5p, [illegible] karyopherin alpha 2 (KPNA2) in glioma tiss[illegible] or cells. The protein expression level of KPN[illegible] was assessed by Western blo[illegible]5-dimeth[illegible] ylthiazol-2-yl)-2,5-diphenyl[illegible]l-3-ium bromide (MTT), flow cyto[illegible]y, and [illegible]answell assays were conducted [illegible]evaluat[illegible]lifera-tion, apoptosis, migrati[illegible]d in[illegible] oma cells, respecti[illegible]onship be-tween miR-499a-5p[illegible] circ_0[illegible]3 or KPNA2 was analyzed by [illegible]nformatics [illegible]ase and confirmed by D[illegible]siferase rep[illegible]analy-ses, respecti[illegible]fects of circ_0079593 silencing in v[illegible] were [illegible]red by a xenograft experime[illegible]

RESU[illegible] Circ_0079593 a[illegible]NA2 were ele-vated i[illegible]oma t[illegible]sues and cells. Loss-of-functio[illegible]perim[illegible] revealed that knockdown of cir[illegible]9593[illegible]mpered [illegible]he progression of gli-oma [illegible]ing pr[illegible]ation, motility and in-ducing [illegible]sis in[illegible]o and declining tumor [illegible]h *in* [illegible]S[illegible]arly, suppression of KP-NA[illegible]peded [illegible]ocess of glioma by inhibit-ing [illegible]iferation, [illegible]otility and increasing apoptosis. [illegible]5p, interacting with KPNA2, was a t[illegible]irc_0079593. In addition, overex-[illegible]ssion of KPNA2 could reverse the effects of [illegible]0079593 knockdown on proliferation, apop-[illegible] migration and invasion of glioma cells. Me[illegible]istically, circ_0079593 mediated prolif-eration, motility and apoptosis of glioma cells by [illegible]ulating KPNA2 expression via sponging miR-[illegible]a-5p.

CONCLUSION[illegible]Circ_0079593 stimulated the p[illegible]logical pr[illegible]ss of glioma via acting as co[illegible]g e[illegible]enous RNA to sponge miR-499a-[illegible]

Key Words:
[illegible]RNA, Circ_0079593, miR-499a-5p, KPNA2, [illegible]ion.

Introduction

Glioma is one of the most common primary intracranial tumors and ranks sixth among all causes leading to cancer-related death worldwide[1,2]. The surgery, chemotherapy and radiotherapy are considered major standard therapies for glioma[3], but the prognosis of glioma patients is still far from satisfactory. The median survival time of glioma patients remains poor[4,5]. Accordingly, it is pivotal to find reliable diagnostic biomarkers and feasible therapeutic targets for glioma.

Circular RNA (circRNA), a loop structure without protein coding potentiality and 5'end cap as well as 3' end ploy A tail[6], has attached much attention due to it can affect gene abundance by sponging miRNA[7,8]. Besides, stability and conservation were typical characteristics of circRNA in human genome[9]. Increasing literature reported that circRNA played a vital role in RNA splicing and transcription, using as an innovative target and biomarker for treatment and prognosis of malignant tumors. For example, as reported by Jin et al[10], circRNA circHIPK3 was a prognostic mark-

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er and therapeutic target in glioma. In addition, circ_0079593 located in chr7 23381682-23383472 was upregulated in glioma patients with larger tumor size and higher World Health Organization grades [11]. Whereas, the exact role of circ_0079593 and its related regulatory mechanism in the process of glioma were rarely uncovered.

Additionally, miRNA, with about 20 nts in length, was classified as non-coding RNA, and could regulate translation and mRNA degradation[12]. Increasing literature demonstrated that miR-499a-5p (aliases as miR-499) exerted its role as a tumor inhibitor in some cancers. Liu et al[13] confirmed that miR-499a-5p was reduced in osteosarcoma tissues by miRNA microarray analysis. Upregulation of miR-499-5p impeded cell proliferation and enhanced apoptosis to repress metastasis of lung cancer[14]. Whereas, the precise role of miR-499a-5p in other cancer diseases was unclear.

Currently, we checked the abundance of circ_0079593, miR-499a-5p, and karyopherin alpha 2 (KPNA2) in glioma tissues and cells. Loss or gain-of function experiments and bioinformatics analyses were carried out to explore the function and regulatory mechanism of circ_00[illegible]93 in glioma cells. Finally, a xenograft resea[illegible] used to assess circ_0079593 function *in vi*[illegible]

Materials and Met[illegible]ds

Human Tissue Sample C[illegible]ectio[illegible]

Thirty human glioma [illegible]sues a[illegible]tched healthy tissues were surg[illegible]rese[illegible] oma patients and vo[illegible]ers [illegible]erwent surgery at The Fifth P[illegible]e's Hospi[illegible] Ji'nan City. All excised tissu[illegible]re timely fr[illegible] liquid nitrogen and p[illegible] -80°C for a[illegible]ng-time preservation [illegible]the fo[illegible]g study. This study was appro[illegible] by the Eth[illegible]mmittee of The Fifth Pe[illegible]s Hospital of Ji'n[illegible]ty and written inform[illegible]consen[illegible]as obtained from all of the recru[illegible]glioma[illegible]ents and volunteers.

Cells C[illegible]

[illegible]e Am[illegible]T[illegible]ulture Collection (ATCC, M[illegible]as, VA[illegible]) provided us with Human glio[illegible] cells (T9[illegible]G and LN229 cells). Human gli[illegible]EB cells) were obtained from the C[illegible] Bank [illegible]he Type Culture Collection of the [illegible]nese Academy of Sciences (Shanghai, China). [illegible]lls were propagated in Dulbecco's Modified Eag[illegible] Medium (DMEM; HyClone, South-Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA), 100 unit ml/L of penicillin (Gibco BRL), and 100 µg ml/L of streptomycin (Gi[illegible] humidified air at 37°C with 5% CO[illegible]

Real-Time Quantitative Poly[illegible]se Chain Reaction (RT-qPCR)

TRIzol reagent (Invit[illegible]d, Carls[illegible]CA USA) was employed to[illegible]ate RNA fro[illegible] ma tissues or cells fo[illegible]ng the[illegible]ndard pr[illegible]col. The quality an[illegible]grity[illegible]he RN[illegible] were checked by Nano[illegible]op[illegible]ermo F[illegible]r Scientific, Waltha[illegible]A, U[illegible]A fter[illegible]s, RNA was reverse[illegible]anscribed [illegible]ementary DNA with [illegible]ript™ RT reag[illegible]it (TaKaRa, Dalian, C[illegible]a) [illegible]Man microRNA Reverse Transcription Kit ([illegible]d Biosystems, Foster City, [illegible]SA). The e[illegible]on of circ_0079593 a[illegible]A2 was quantifi[illegible]d by SYBR Premix [illegible]Taq II (TaKaRa) and normalized to glyceraldehyde-3-phos[illegible]e dehydrogenase (GAPDH), [illegible]e the expre[illegible]n ofmiR-499a-5p was standa[illegible]d to en[illegible]nous small nuclear RNA U6 usi[illegible]d.

The se[illegible]ces of primers were as follows:

Circ_0079593 (Divergent primers; Forward, [illegible]GACCCACTTCAGGGAT-3'; Reverse, [illegible]CATGTCTCAACATTGCCT-3');

circ_0079593 (Convergent primer; Forward, 5'-ACTGCACGGGAAACCCATAG-3'; Reverse, 5'-ACTATCCAGCACCTCCCACT-3');

miR-499a-5p (Forward, 5'-GCCGAGTTAAGACTTGCAGTGA-3'; Reverse, 5'-CTCAACTGGTGTCGTGGA-3');

KPNA2 (Forward, 5'-ATTGCAGGTGATGGCTCAGT-3'; Reverse, 5'-CTGCTCAACAGCATCTATCG-3');

GAPDH (Forward, 5'-TCCCATCACCATCTTCCAGG-3'; Reverse, 5'-GATGACCCTTTTGGCTCCC-3');

U6 (Forward, 5'-AACGCTTCACGAATTTGCGT-3'; Reverse, 5'-CTCGCTTCGGCAGCACA-3').

Western Blot Assay

The glioma tissues or cells were lysed using lysis buffer added with proteinase inhibitor (Invitrogen). The protein concentration was quantified by the bicinchoninic acid (BCA) protein assay (Bio-Rad, Hercules, CA, USA). Then, equal amounts of proteins were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and shifted onto a polyvinylidene difluoride (PVDF; Thermo Fisher Scientific, Waltham, MA, USA). Membranes were enshrouded with 5% skim

milk solution and then interacted with the primary antibodies against KPNA2 (1:1000 dilution, Boster, Wuhan, China), GAPDH (1:3000 dilution, Boster) as a reference control. After being washed, membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000 dilution, Boster) at room temperature. After that, antibody binding was visualized and analyzed with enhanced chemiluminescence (ECL) Western Blotting Detection Kit (Solarbio, Beijing, China) and Image J software (National Institutes of Health, Bethesda, MD, USA), respectively.

Cell Transfection

Specific small interfering RNA against circ_0079593 (si-circ_0079593) and its scrambled control (si-NC), overexpressed plasmid of circ_0079593 (circ_0079593) or and KPNA2 (pcDNA-KPNA2) and their negative control (pcDNA-NC), miR-499a-5p mimic (miR-499a-5p) and its negative control (miR-NC), specific short hairpin RNA (shRNA) against KPNA2 (sh-KPNA2) or against circ_0079593 (sh-circ_0079593) and their negative control (sh-NC), were bought from Sangon (Shanghai, China). T98G and LN22[illegible] were transfected with above oligonucleo[illegible] vectors in combination or alone utilizing [illegible]o-fectamine 2000 (Invitrogen, Carlsbad, CA, [illegible] in accordance with the instructions of manu[illegible] turer.

3-(4,5-Dimethylthiazol-[illegible]-2,5-[illegible]henyl-2H-Tetrazol-3-ium Br[illegible]e (MT[illegible]say

The MTT assay was [illegible]ted cell viability of T98[illegible]d L[illegible]lls. Briefly, cells were suspend[illegible] 200 µL [illegible]edium and planted into 96-[illegible]ulture plate[illegible]10^3 cells/well). After in[illegible]37°C for spec[illegible]ed time, MTT (Therm[illegible]sher S[illegible]c) was used to interact cells fo[illegible]other 4 h at [illegible]. After the removal of the s[illegible]natant, 150 µL o[illegible]ethyl sulfoxide (DMS[illegible]was add[illegible] to each well. We recorded the pro[illegible]tion o[illegible]s by monitoring optical density a[illegible]of 490[illegible]on microplate reader (Applie[illegible]stems[illegible]ter City, CA, USA) at 0 [illegible]h, 48[illegible] 7[illegible] respectively.

Flo[illegible]Cytometry

[illegible]229 cells were washed with phos[illegible]-buffer[illegible]saline (PBS) three times after trans[illegible]ion and collected with trypsin for apoptotic [illegible]is. Cells were adjusted to single cell suspension[illegible] interacted with 5 µL of Annexin V labeled with fluorescein isothiocyanate (FITC) and 5 µL of propidium iodide (PI) at room temperature for 30 min in the dark. The apoptotic cells we[illegible]monitored by flow cytometry (Becton-Dic[illegible] Jose, CA, USA) as instructed by the [illegible]ufacture[illegible] Annexin V positive cells served a[illegible]optotic cells.

Transwell Assay

The 24-well transwell c[illegible]ber with[illegible]tri[illegible] gel (BD Biosciences, F[illegible]in Lakes, NJ, [illegible] or adhered with Mat[illegible], was [illegible]loyed to [illegible]-amine cell migratio[illegible]d inva[illegible], respe[illegible]tively. T98G and LN229 [illegible]ells[illegible]vated i[illegible]upper chamber with [illegible]n-free [illegible]um, [illegible]e low-er chamber [illegible]filled with [illegible]ontaining 10% fetal b[illegible]rum (FBS) a[illegible]rients. Cells on the b[illegible]side[illegible] membrane were scraped with cotton swab a[illegible]ubation 24 h, migrated or in[illegible]cells were fi[illegible]ith 95% ethanol and d[illegible]0.1% crystal vio[illegible]t. The migration and [illegible]sion cells were photographed and computed [illegible]ve randomly [illegible]ected visual fields with a mi-[illegible]cope (Olym[illegible] Tokyo, Japan) and Image J s[illegible]e (Natio[illegible]nstitutes of Health, Bethesda, MD[illegible]

Dual-Luciferase Reporter Assay

[illegible]irect interactions among circ_0079593, [illegible]a-5p, and 3'untranslated region (UTR) of KPNA2 were predicted by starBase2.0 (http://starbase.sysu.edu.cn/starbase2/). The luciferase reporters of wild type (WT-circ_0079593 and KPNA2-WT) containing miR-499a-5p interacting sites and matched mutant type (MUT-circ_0079593 and KPNA2-MUT) were constructed and co-transfected into T98G and LN229 cells with miR-NC, miR-499a-5p, miR-499a-5p+pcDNA-NC, or miR-499a-5p+circ_0079593. At 48 h after transfection, the luciferase activity was detected with Dual-Luciferase Assay Kit (GeneCopoeia, Rockville, MD, USA) with Renilla luciferase activity as a reference control.

Xenograft Experiment

Animal experiment was approved by the Institutional Animal Care and Use Committee of The Fifth People's Hospital of Ji'nan City. 10 BALB/c mice (females; 4 weeks of age) were obtained from Henan Experimental Animals Centre (Zhengzhou, China) and were divided into two groups. LN229 cells were stably transfected with sh-circ_0079593 to impede circ_0079593 expression, sh-NC as a reference control. A volume of 200 µL of PBS containing the transfected LN229 cells or control (2×10^6/0.2 mL of PBS) was injected into the right

flanks of the mice. Tumor growth was monitored after post-transcription 7 d, and size was calculated using $V = 1/2\times ab^2$ method (length (a) and width (b) length of the tumor). 27 d after injection, mice were killed by cervical dislocation and tumors were removed for the weight detection and analyzed by western blot and RT-qPCR.

Statistical Analysis

All data were shown as mean ± standard deviation from three independent experiments and $p < 0.05$ indicated a statistically significant difference. The relationship between circ_0079593 and miR-499a-5p orKPNA2 expression was analyzed with Pearson's correlation assay. The Student's *t*-test or one-way analysis of variance was used to analyze significant difference for two groups or among multiple groups using SPSS 17.0 (SPSS Inc., Chicago, IL, USA), respectively, following by Tukey's test.

Results

Circ_0079593 and KPNA2 Were Highly Expressed in Glioma Tissues

Initially, RT-qPCR and Western blot were performed to quantify the expression level of circ_0079593 and KPNA2 in glioma tissues and matched normal tissues. We found circ_0079593 was dramatically strengthened in glioma tissues than that in normal tissues (Figure 1B). Similarly, we also found that the mRNA expression level of KPNA2 was elevated in glioma tissues in contrasted to healthy tissues (Figure 1F). Likely, the protein expression level of KPNA2 was intensified, as demonstrated by Western blot assay (Figure 1C-1D). Results of Pearson correlation analysis uncovered that circ_0079593 was positively correlated with KPNA2 expression in glioma tissues (Figure 1E). Together, the data indicated that circ_0079593 and KPNA2 may played important roles in glioma.

Depletion of Circ_0079593 Retarded Proliferation of Glioma

The RT-qPCR analysis confirmed that a higher level of circ_0079593 existed in T98G and LN229 cells when compared with HEB cells (Figure 2A). Subsequently, T98G and LN229 cells were transfected with si-circ_0079593 or si-NC. Analysis of interference efficiency was checked using RT-qPCR assay, revealing a low value for circ_0079593 in si-circ_0079593 group in contrast to si-NC group (Figure 2B). MTT assay suggested that repression of circ_0079593 in T98G and LN229 cells led to an evident reduction of cell viability (Figure 2C-2D). Inversely, an obvious increase of cell apoptosis was triggered in T98G and LN229 cells transfected with si-circ_0079593 (Figure 2E). The functional impacts of circ_0079593 downregulation on migration and invasion of glioma cells were investigated by transwell assay. Transfection of si-circ_0079593 into T98G and LN229 cells declined the number of migration and invasion cells in contrast to that in transfection of si-NC (Figure 2F-2G). These findings revealed the inhibition of circ_0079593 repressed the progress of glioma by attenuating proliferation, migration and invasion but expedited apoptosis of glioma cells.

KPNA2 Silencing Hampered Proliferation, Migration and Invasion but Facilitated Apoptosis in Glioma Cells

Our findings also indicated that KPNA2 was remarkably increased in glioma cells (T98G and LN229 cells) compared to HEB cells, as presented in Figure 3A-3B. Besides, in order to probe the function of KPNA2 in glioma cells, we mitigated the expression of KPNA2 in T98G and LN229 cells by transfecting sh-KPNA2, using sh-NC as reference control. The RT-qPCR and Western blot assays elucidated that KPNA2 level was enormously blocked in T98G and LN229 cells transfected with sh-KPNA2in contrast to cells transfected with sh-NC (Figure 3C-3D). Next, MTT analysis was executed to further assess the function of KPNA2 on glioma cell proliferation. The result exhibited that knockdown of KPNA2 constrained glioma cell proliferation (Figure 3E). Meanwhile, apoptosis rate was enormously accelerated in T98G and LN229 cells transfected with sh-KPNA2 compared to sh-NC group (Figure 3F). The transwell assay confirmed that migration and invasion abilities of T98G and LN229 cells were evidently limited in T98G and LN229 cells transfected with sh-KPNA2 in comparison with sh-NC group (Figure 3G-3H). In summary, above data suggested that knockdown of KPNA2 hampered proliferation, migration and invasion but induced apoptosis in glioma cells.

Circ_0079593 Regulated Proliferation, Apoptosis, Migration and Invasion of Glioma Cells by Affecting KPNA2 Expression

We noticed that the expression level of circ_0079593 in T98G and LN229 cells transfected with circ_0079593 was apparently ag-

grandized with RT-qPCR assay (Figure 4A). The relationship between circ_0079593 and KPNA2 was further explored (Figure 4B-4E). A marked suppression of KPNA2 expression was observed in T98G and LN229 cells transfected with si-circ_0079593 when compared to si-NC group, while overexpression plasmid of circ_0079593 led to an inverse trend. Moreover, transfection efficiency of KPNA2 was checked by RT-qPCR and Western blot analyses, indicating KPNA2 was greatly elevated in T98G and LN229 cells transfected with pcDNA-KPNA2 (Figure 4F-4G). Whether circ_0079593 mediated glioma cells progression, apoptosis, migration and invasion via regulating KPNA2 expression was analyzed with MTT, flow cytometry, and transwell analyses, correspondingly. Furthermore, T98G and LN229 cells were transfected with si-NC, si-circ_0079593, si-circ_0079593+[illegible] or si-circ_0079593+pcDNA-KPN[illegible] MTT a[illegible] say displayed that circ_007959[illegible]encing im-peded cell proliferation, while [illegible]ction was mitigated by enhancement of KP[illegible] T98G and LN229 cells (Figure [illegible]I). The [illegible] flow cytometry assay [illegible]osed that do[illegible] ulation of circ_0079[illegible] drasti[illegible] facilita[illegible] apoptosis, while thi[illegible]motio[illegible]ect w[illegible] elim-inated by trans[illegible]pcDN[illegible]NA2 (Figure 4J-4K) [illegible]nswe[illegible]ysis r[illegible]ed that circ_0079592[illegible]encing sub[illegible]hampered cell migra[illegible] invasion, [illegible]as KPNA2 upregula[illegible] co[illegible]ted this change in T98G

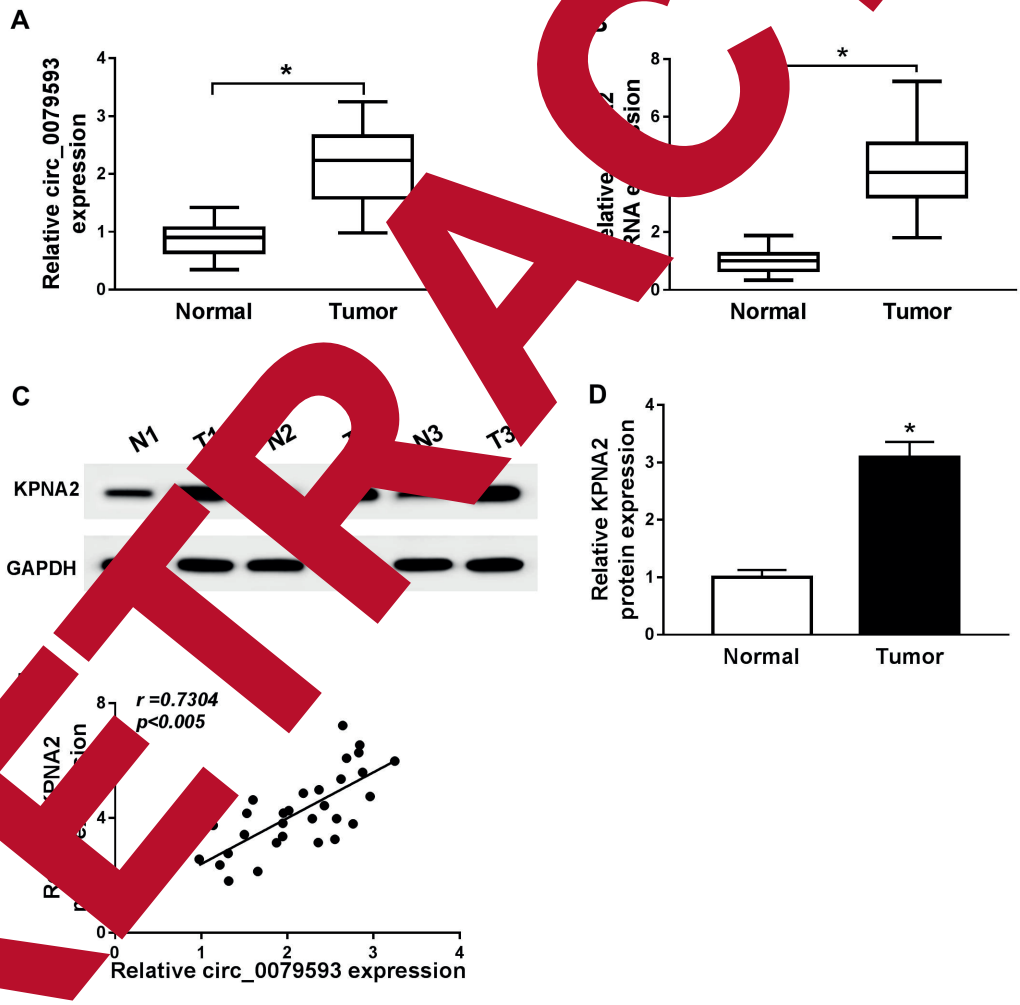

Figure 1. The expression level of circ_0079593 and KPNA2 in glioma tissues. **(A-B)** The RT-qPCR assay was used to analyze the abundance of circ_0079593 and KPNA2 in glioma tissues and matched healthy tissues. **(C-D)** The protein expression level of KPNA2 in glioma tissues and matched healthy tissues was measured by Western blot assay. **(E)** The relationship between circ_0079593 and KPNA2 was examined by Pearson's correlation analysis. *p<0.05.

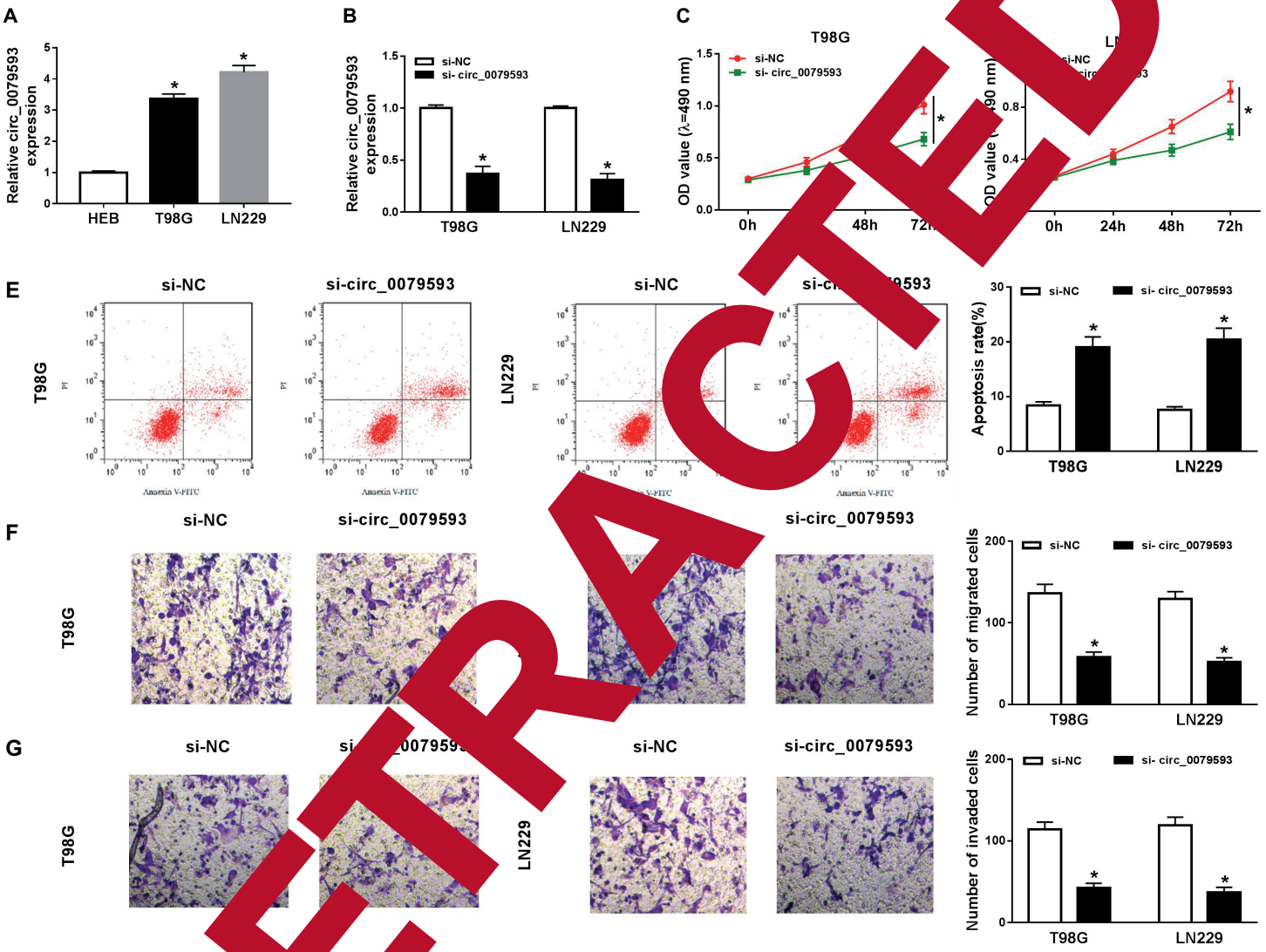

Figure 2. The effects of circ_0079593 si[illegible]on pr[illegible]ation, apoptosis, migration and invasion of glioma cells. **(A)** The RT-qPCR was applied to determine circ_0079593 expression in HEB, T98G, and [illegible]lls. **([illegible])** [illegible]nd LN229 cells were transfected with si-circ_0079593 or si-NC. **(B)** The interference efficiency of si-circ_0079593 was checked by RT-qPCR in T98[illegible]d L[illegible] cells a[illegible]nsfection. **(C-D)** MTT assay was employed to detect cell viability of transfected T98G and LN229 cells. **(E)** Apoptosis of T98G and LN229 cells wa[illegible]nitored [illegible]low cytom[illegible]try assay. **(F-G)** Effects of si-circ_0079593 on the migration and invasion of T98G and LN229 cells were estimated with by transwell assay (100 X) [illegible]05.

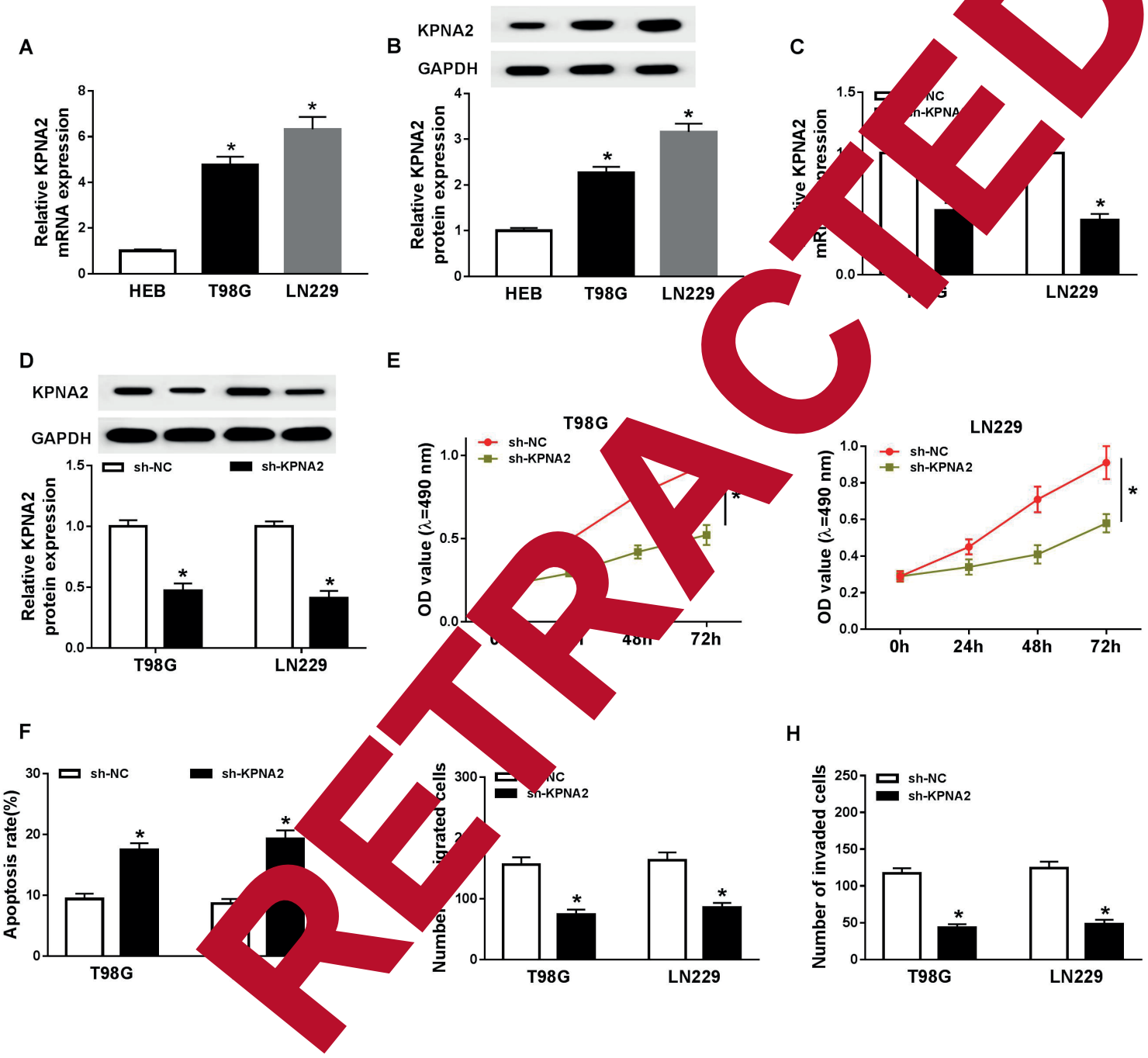

Figure 3. The impacts of KPNA2 silencing on proliferation, apoptosis, migration and invasion of glioma cells. **(A-B)** The mRNA and protein expression levels of KPNA2 were assessed in HEB, T98G, and LN229 cells by RT-qPCR and Western blot assays, respectively. **(C-H)** T98G and LN229 cells were transfected with sh-KPNA2 or sh-NC. **(C-D)** The RT-qPCR and Western blot assays were performed to evaluate the expression level of KPNA2 in T98G and LN229 cells. **(E)** The cell viability of T98G and LN229 cells was determined with MTT assay. **(F)** Effect of sh-KPNA2 on apoptosis of T98G and LN229 cells was tested with flow cytometry assay. **(G-H)** The transwell assay was conducted to analyze migration and invasion of T98G and LN229 cells. *$p< 0.05$.

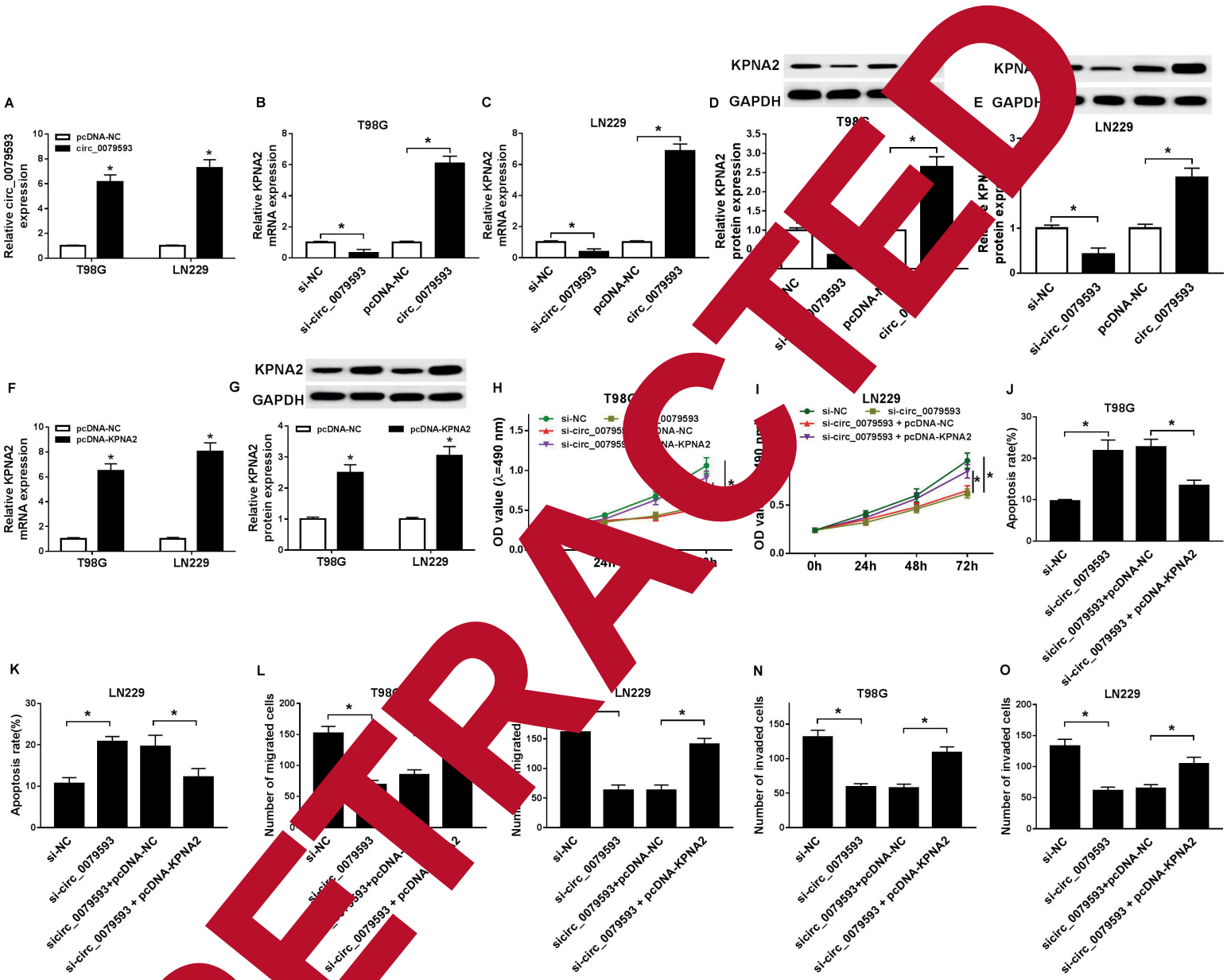

Figure 4. **(A)** The expressi vel o 007959 98G and LN229 cells transfected with pcDNA-NC or circ_0079593 was measured with RT-qPCR assay. **(B-E)** RT-qPCR and Western blot analyses wer oduced xamine expression level of KPNA2 in T98G and LN229 cells transfected with si-NC, si-circ_0079593, pcDNA-NC or circ_0079593. **(F)** The transfection efficiency of 2 was qPCR and Western blot analyses in T98G and LN229 cells. **(H-O)** T98G and LN229 cells were transfected with si-NC, si-circ_0079593, si-circ_0079593+ pcDNA-N _0079593 + pcDNA-KPNA2. **(H-I)** Cell viability of transfected T98G and LN229 cells was determined by MTT assay. **(J-K)** Flow cytometry was used to detect apoptosis of T98 N229 cells after transfection. **(L-O)** The cell capabilities of migration and invasion were estimated with transwell analysis post-transfection. *$p < 0.05$.

and LN229 cells (Figure 4L-4O). Thus, we concluded circ_0079593 mediated proliferation, apoptosis, migration and invasion through affecting KPNA2 expression in glioma cells.

Circ_0079593 Negatively Regulated MiR-499a-5p Expression in Glioma Cells

The complementary binding sequences and corresponding mutant regions for circ_0079593 on miR-499a-5p were displayed in Figure 5A. T98G and LN229 cells were co-transfected miR-499a-5p mimic or miR-NC along with WT-circ_0079593 or MUT-circ_0079593 reporter. The result of Dual-Luciferase reporter assay confirmed that miR-499a-5p mimic declined luciferase activity of WT-circ_0079593 but didn't change luciferase activity of MUT-circ_0079593, suggesting that circ_0079593 directly interacted with miR-499a-5p (Figure 5B-5C). Also, miR-499a-5p was downregulated in glioma tissues and cells when compared with healthy tissues as well as HEB cells, respectively (Figure 5D-5E). Additionally, the miR-499a-5p was negatively correlated with circ_0079593 expression in glioma tissues (Figure 5F). As shown in Figure 5G-5H, si-circ_0079593 promoted miR-499a-5p expression in T98G and LN229 cells; accordingly, the expression level of miR-499a-5p was decreased in T98G and LN229 cells transfected with circ_0079593. Finally, introduction of miR-499a-5p in T98G and LN229 cells drastically augmented the expression of miR-499a-5p compared with the control group (Figure 5I). These results implied that miR-499a-5p was a target of circ_0079593 in glioma cells.

KPNA2 was Identified as a Target of MiR-499a-5p in Glioma Cells

The interaction between KPNA2 and miR-499a-5p was analyzed with bioinformatics soft starBase2.0 (Figure 6A). In addition, Dual-Luciferase report assay detected that miR-499a-5p mimic reduced the luciferase activity of KPNA2-WT reporter vector but not that of KPNA2-MUT reporter vector; importantly, miR-499a-5p in combination with circ_0079593 abolished the reduction (Figure 6B-6C). The RT-qPCR and Western blot assays were recruited to analyze the mRNA and protein expression levels of KPNA2 in T98G and LN229 cells transfected with miR-NC, miR-499a-5p, NA-NC, or circ_0079593. Upregulation of circ_0079593 abrogated overexpression of miR-499a-5p-induced decline of KPNA2 level (Figure 6D-6G). All results suggested that circ_0079593 regulated KPNA2 expression in glioma cells by sponging miR-499a-5p.

Suppression of Circ_0079593 Constrained Tumor Growth in Vivo

Given that inhibition of circ_0079593 suppressed proliferation but expedited apoptosis of glioma cells, LN229 cells were stably transfected with sh-circ_0079593 and subcutaneously injected into mice to construct a tumor xenograft model, sh-NC as a reference control. Tumor growth curves and weights were shown in Figure 7A-7B: knockdown of circ_0079593 resulted in a great decrease in tumor volume and weight when compared with sh-NC group. In addition, circ_0079593 was detected and miR-499a-5p was increased in dissected tumor tissues with RT-qPCR assay (Figure 7C-7E). A significant decline in KPNA2 was observed in the tumor xenografts of circ_0079593 group with respect to sh-NC group, whether in mRNA or protein levels (Figure 7F). Taken together, circ_0079593 silencing retarded tumor growth *in vivo*.

Discussion

In this study, we identified that circ_0079593 was associated with glioma development, which was a reliable biomarker for diagnosis and treatment of glioma patients.

It has been widely acknowledged that competitive endogenous RNAs (ceRNAs) theory was existed and functioned with key roles in the initiation and development of cancers[15,16]. Li et al[17] reported that Hsa_circ_0046701 acted in a critical function in glioma pathogenesis by regulating miR-142-3p/integrin subunit beta 8 axis via acting as molecule sponge to miR-142-3p. Additionally, circRNA played a vital role in tumorigenesis via serving as oncogene or tumor suppressor, providing potential biomarker for prognostic of cancer patients, as circ_0001649 was functioned as a rational target for cholangiocarcinoma-related therapeutic[18]. Whereas, researches were rare for investigation of the relationship between circ_0079593 and glioma process previously. Our results uncovered that circ_0079593 was strikingly amplified in glioma tissues and cells. As we expected, downregulation of circ_0079593 declined cell viability and capabilities of migration and invasion but accelerated apoptosis of glioma cells, indicating that the suppression of circ_0079593 retarded the process of glioma. This was similar with the results reported by Qu et al[11] that showed circ_0079593 facilitated growth and invasion of glioma cells.

Afterwards, bioinformatics and Dual-Luciferase reporter analyses confirmed that circ_0079593

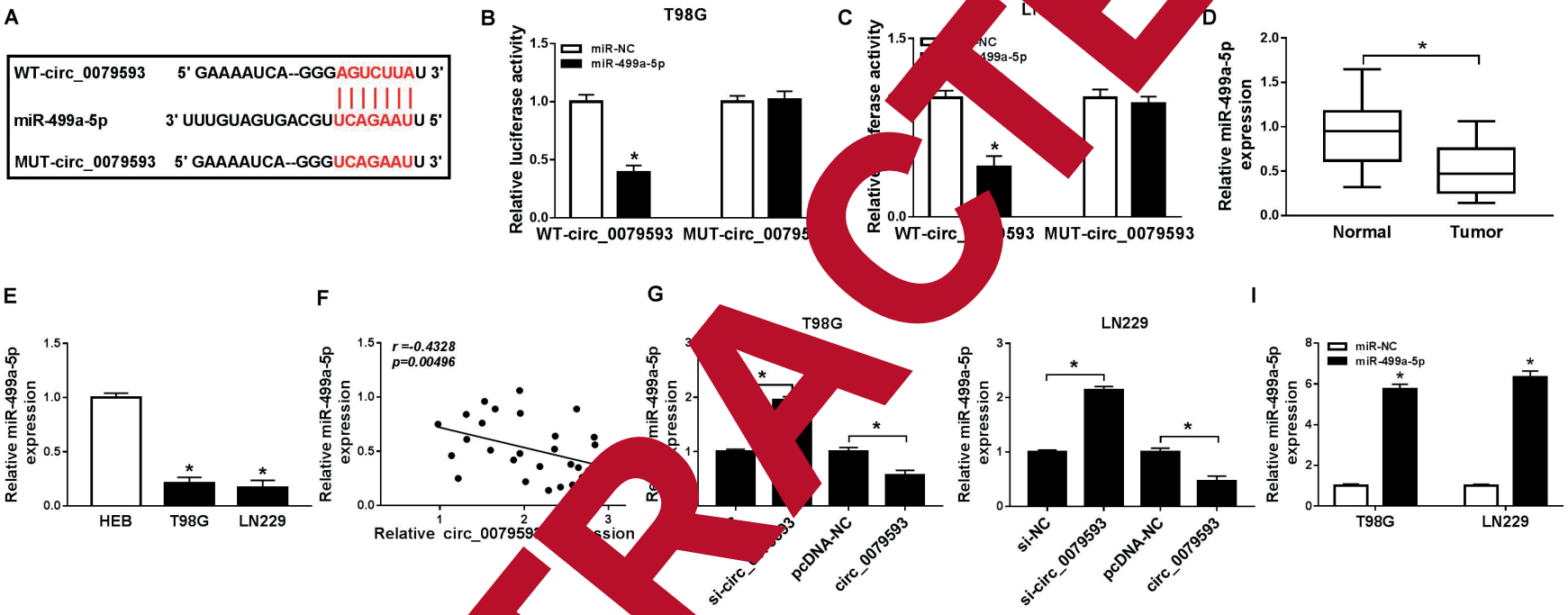

Figure 5. MiR-499a-5p was a direct target of circ_0079593 in glioma cells. **(A)** Binding region between miR-499a-5p and circ_0079593 was predicted by starBase2.0. **(B-C)** Luciferase activity of WT-circ_0079593 or MUT-circ_0079593 reporter in T98G and LN229 cells following the transfection of miR-499a-5p mimic or miR-NC was assessed by dual-luciferase reporter assay. **(D-E)** The RT-qPCR assay was applied to evaluate the expression level of miR-499a-5p in glioma tissues and matched healthy tissues as well as in HEB, T98G, and LN229 cells. **(F)** The Pearson's correlation assay was carried out to test the relationship between circ_0079593 and miR-499a-5p in glioma tissues. **(G-I)** The expression level of miR-499a-5p in T98G and LN229 cells transfected with si-NC, si-circ_0079593, pcDNA-NC, circ_0079593, miR-499a-5p, or miR-NC. *$p< 0.05$.

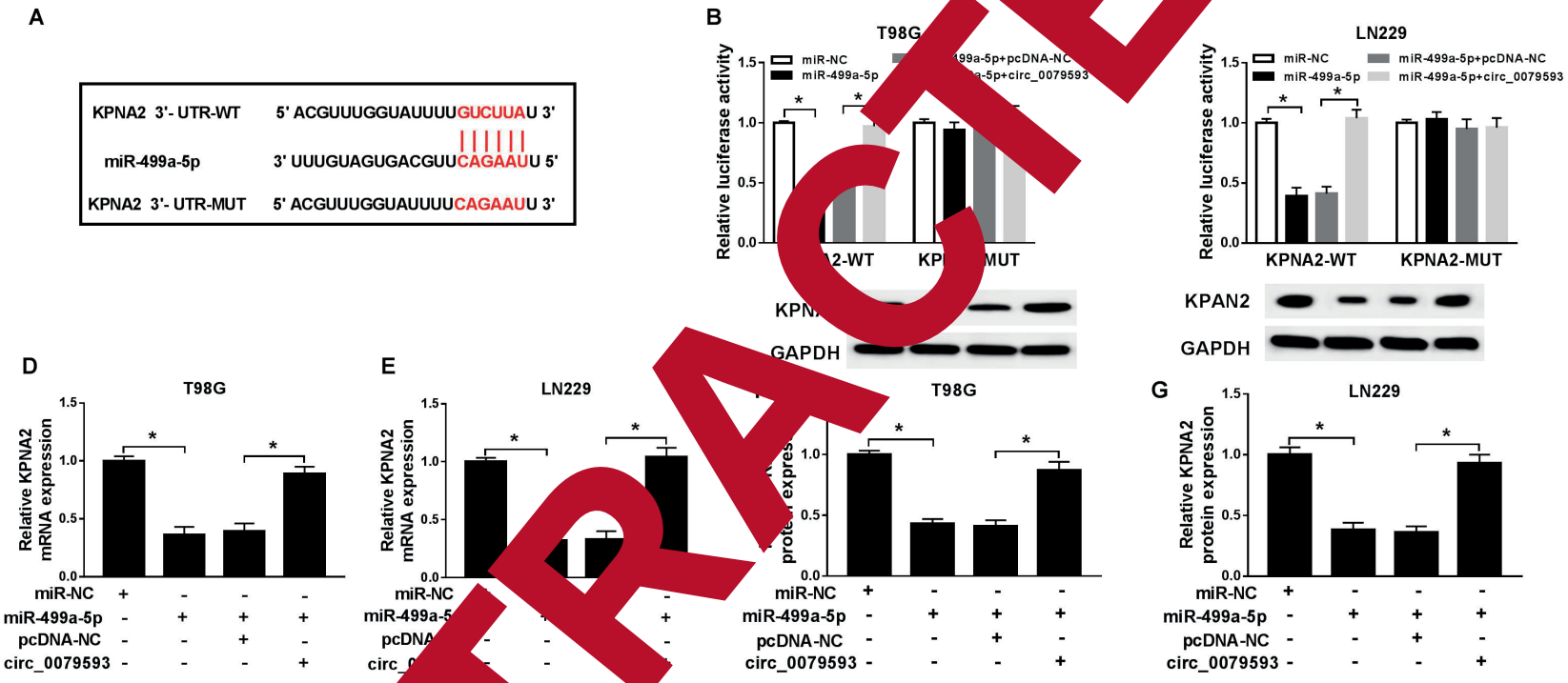

Figure 6. MiR-499a-5p regulated KPNA2 expression in gl[illegible]ells. **(A)** The starBase2.0 was employed to predict binding sites between miR-499a-5p and 3'UTR of KPNA2. **(B-C)** The relationships among miR-499a-5[illegible]PNA2, and ci[illegible]9593 were verified by dual-luciferase reporter assay. **(D-G)** The mRNA and protein expression levels of KPNA2 were estimated in T98G and LN2[illegible]ells transfected with[illegible]R-NC, miR-499a-5p, miR-499a-5p+pcDNA-NC, or miR-499a-5p+circ_0079593 by RT-qPCR and Western blot assays, respectively. *p< 0.05.

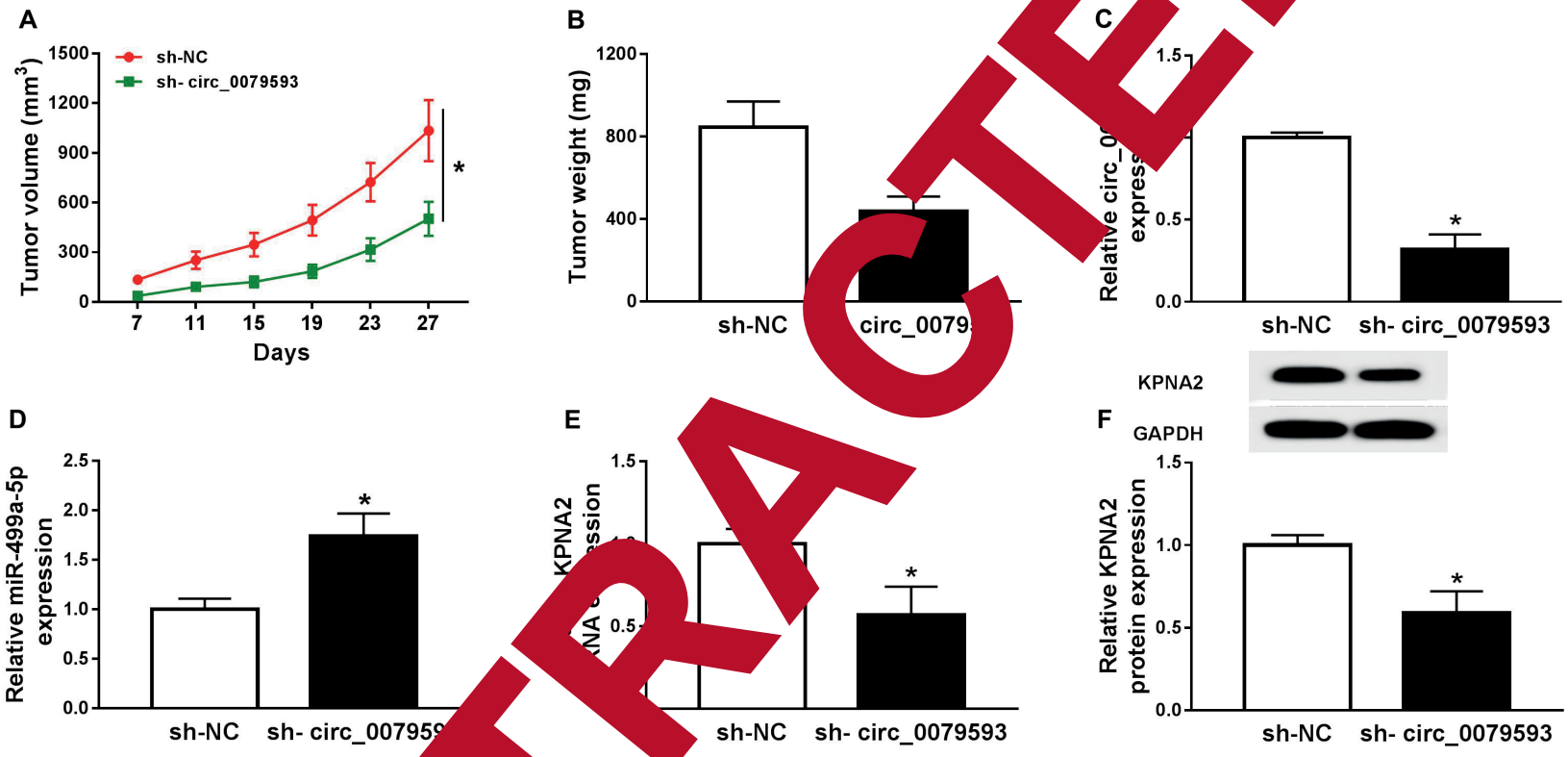

Figure 7. Circ_0079593 silencing impeded t[illegible] growth [illegible] **(A)** The volume of tumors was examined with a caliper. **(B)** The tumor weight was weighed after tumor dissection from nude mice. **(C-D)** The expressi[illegible]vels of circ_00[illegible]and miR-499a-5p in dissected tumor tissues were measured with RT-qPCR assay. **(E-F)** The RT-qPCR and western blot assays were applied to exami[illegible]PNA2 level in remo[illegible]d tumor tissues. *$p < 0.05$.

could act as a molecular sponge for miR-499a-5p. Meanwhile, our data displayed that miR-499a-5p was decreased in glioma tissues and cells and negatively correlated with circ_0079593 in glioma tissues. Analogously, Zong et al[19] proved that miR-499a-5p was a target of secretory carrier membrane protein 1 in glioma and exerted tumor-suppressive function in glioma. However, abundance of circulating miR-499 in plasma of patients with acute myocardial infarction was remarkably elevated compared with healthy control group [20]. The results were consistent with those of Olivieri et al[21]. In addition, enhancement of miR-499-5p stimulated cell metastasis of colorectal cancer [22]. It is worth to investigate the expression level of miR-499 in special tissues in further studies.

KPNA2, a member of the Karyopherin α family, was highly expressed in glioma tissues and cells. Analogously, KPNA2 was overexpressed in colorectal cancer[23], gallbladder cancer[24], and epithelial ovarian carcinoma[25]. In view of miRNA regulated gene expression via targeting 3'UTR of gene in tumorigenesis, we guessed that KPNA2 was a target of miR-499a-5p and verified it with bioinformatics and Dual-L[illegible] erase reporter analyses. Suppressive [illegible] of KPNA2 silencing on the growth and [illegible]stasis were observed in glioma cells. Our re[illegible] showed that knockdown of KPNA2 repres[illegible] growth and mobility but promot[illegible] poptosis [illegible] glioma cells. Consistently, [illegible] an oncogene role was observed [illegible]revio[illegible]esults[26]. Moreover, the expressio[illegible]el of K[illegible]A2 was remarkably repressed b[illegible]R-49[illegible] markedly increased [illegible]irc_[illegible]5 in glioma cells, suggesting [illegible]c_0079[illegible]R-499a-5p/KPNA2 network [illegible]losely assoc[illegible]ith development of [illegible]

Conclusions

W[illegible]at cir[illegible]79593 and KPNA2 were up[illegible]ed an[illegible]ated to the progression [illegible]ioma. [illegible] experiments suggested tha[illegible]c_007[illegible]ilencing or knockdown of KP[illegible] impeded[illegible]he process of glioma through de[illegible]liferation, migration and inva[illegible] but [illegible]asing apoptosis. Mechanistically, [illegible]_0079593 contributed to glioma progression [illegible]h mediating miR-499a-5p/KPNA2 axis ind[illegible]g a new biomarker and therapeutic approach for glioma.

Conflicts of interest

The authors declare that they have no conf[illegible] terests.

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