Nuclear Dbf2-related Kinase 1 functions as tumor suppressor in glioblastoma by phosphorylation of Yes-associated protein

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Abstract

Background: The Nuclear Dbf2-related (NDR1) kinase is a member of the NDR/LATS family, which was a supplementary of Hippo pathway. However, whether NDR1 could inhibit glioblastoma (GBM) growth by phosphorylating Yes-associated protein (YAP) remains unknown. Meanwhile, the role of NDR1 in GBM was not clear. This study aimed to investigate the role of NDR1-YAP pathway in GBM.

Methods: Bioinformation analysis and immunohistochemistry (IHC) were performed to identify the expression of NDR1 in GBM. The effect of NDR1 on cell proliferation and cell cycle was analyzed utilizing CCK-8, clone formation, immunofluorescence and flow cytometry, respectively. In addition, the xenograft tumor model was established as well. Protein interaction was examined by Co-immunoprecipitation and immunofluorescence to observe co-localization.

Results: Bioinformation analysis and IHC of our patients' tumor tissues showed that expression of NDR1 in tumor tissue was relatively lower than that in normal tissues and was positively related to a lower survival rate. NDR1 could markedly reduce the proliferation and colony formation of U87 and U251. Furthermore, the results of flow cytometry showed that NDR1 led to cell cycle arrest at the G1 phase. Tumor growth was also inhibited in xenograft nude mouse models in NDR1-overexpression group. Western blotting and immunofluorescence showed that NDR1 could integrate with and phosphorylate YAP at S127 site. Meanwhile, NDR1 could mediate apoptosis process.

Conclusion: In summary, our findings point out that NDR1 functions as a tumor suppressor in GBM. NDR1 is identified as a novel regulator of YAP, which gives us an in-depth comprehension of the Hippo signaling pathway.

Keywords: Glioblastoma; Hippo signaling pathway; Nuclear Dbf2-related; Yes-associated protein

Introduction

Glioma is the most common primary malignant brain tumor, and originates from glial cells or glial precursor cells. Glioblastoma (GBM, WHO grade IV) is one of the most common and most malignant primary tumors in the brain. Despite the current efforts of medical investigations, the prognosis of GBM is still poor. The 5-year survival rate of GBM is <5%[1]; the overall survival of patients in clinical studies is between only 14.6 to 26.3 months.[2] Besides, the pathogenesis of GBM is unique and complex with a large number of gene mutations and complex signal pathway changes.[3] Therefore, improving glioma therapy is the focus and challenge of neurosurgery research.

Yes-associated protein (YAP) is a transcriptional coactivator that promotes tissue and organ growth.[4] It is a core effector of the Hippo signaling pathway. Mammalian sterile-20-like 1/2 (MST1/2) phosphorylates large tumor suppressor 1/2 (LATS1/2), which in turn phosphorylates YAP, leading to cytoplasmic retention and loss of function.[5] Accumulating evidence also shows that overexpression (OE) of YAP/TAZ is observed in many cancer types including glioma, indicating that YAP/TAZ not only promotes organ growth, but also plays a key role in tumor proliferation.[6,7] High YAP or TAZ activity enables the cell to escape contact inhibition and anoikis and supports anchorage-independent growth.[8] This result indicates that the regulation of YAP is significant for the inhibition of glioma growth. At present, the upstream regulator of YAP is still being investigated. Nuclear Dbf2-related (NDR1), also known as serine/threonine kinase 38 or STK38), drew our attention because of its similarity to LATS.

NDR1 is a member of the NDR/LATS family including NDR1, NDR2 (or STK38L), large tumor suppressor-1 (LATS1) and large tumor suppressor-2 (LATS2), which is a member of the AGC (protein kinase A/G/C) group of the

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serine/threonine kinase family.[9] The NDR protein kinase is highly conserved and is nearly universally expressed in many different species.[10] NDR/LATS kinase is essential for cell growth, metabolism, proliferation, and survival of many unicellular or multicellular organisms.[11] It is generally believed that NDR has similar effects like LATS acting as a tumor suppressor protein in the Hippo pathway.[12] However, there is also evidence that NDR can promote tumor growth. As an important supplement to the Hippo pathway, NDR1 has attracted much attention in the past decade. However, the role of NDR1 in glioma remains unknown. Now that NDR1 is known to have a similar function as LATS, it is also unclear whether NDR1 may exert a tumor suppressor function by combining with YAP.

Methods

Ethics

Our research was approved by the Medical Science Research Ethics Committee of our hospital and followed the guidance of World Medical Association *Declaration of Helsinki*-Ethical Principles for Medical Research Involving Human Subjects. Considering our specimens are all obtained from the pathology department, the ethics committee believes that our research does not require additional patient informed consent, and only needs to pay attention to avoid unauthorized disclosure patients' information. As a result, there is no relevant ethical ID.

Study design

All the GBM specimens were collected from the department of pathology of Peking University Third Hospital. Including criteria: The pathological diagnosis was WHO IV level from January 1, 2017 to December 31, 2018. All the patients were followed up for at least 1 year by telephone or outpatient. All the clinical information of 158 patients was collected from the Electronic Medical Record Information System. They were divided into two groups by integrated score of immunohistochemistry (IHC) of NDR1 including low expression group and high expression group [Supplementary Digital Content, Figure 1, http://links.lww.com/CM9/A696]. Clinical information of the two groups was compared to identify the differences in terms of prognosis and other clinical items.

Bioinformatic analysis of the ONCOMINE microarray database and SurvExpress database

Expression levels of NDR1 (STK38) in GBM were analyzed via ONCOMINE microarray datasets (https://www.oncomine.org). These datasets play a powerful role in screening differentially expressed genes (DEGs) between tumor and normal tissues. By searching "STK38," "GBM," "Cancer *vs*. Normal Analysis," and "mRNA," three datasets were acquired. The survival information of patients with lower and higher STK38 expression was obtained from SurvExpress: An Online Biomarker Validation Tool and Database for Cancer Gene Expression Data Using Survival Analysis (http://bioinformatica.mty.itesm.mx/SurvExpress).

Cell culture

Human GBM cancer cell lines U87 MG and U251 were purchased from the American Type Culture Collection (Manassas, VA, USA). U87 MG cells were cultured in Minimum Essential Medium-α supplemented with 10% fetal bovine serum (FBS, BI, Israel) and 1% nonessential amino acids (NEAAs, Gibco, USA). U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% FBS. Cells were maintained at 37°C in a humidified incubator with a constant airflow of 5% CO_2.

Recombinant of lentiviruses

Recombinant lentivirus OE RNA targeting the NDR1 sequence (Lv-OE-NDR1) and a lentivirus control sequence (Lv-Con) with green fluorescent protein (GFP) were purchased from Jikai (Shanghai, China). The mRNA sequence adopted in our study was NM_007271.4.

Cell infection with lentivirus

U87 MG and U251 cells were seeded in 6-well plates with lentivirus; cells were cultured in complete medium containing virus for 16 h, and then the medium was replaced with normal medium. Observed by a fluorescence microscope, GFP-positive cells were used to estimate infection efficiency. A culture medium containing puromycin (Solarbio, Beijing, China) was used to kill unsuccessfully transfected cells.

Western blot assay

U87 MG and U251 cells with stable NDR1-OE and control cells were collected and lysed in Radio Immunoprecipitation Assay buffer (Solarbio, Beijing, China) and western blot assays of equal volumes of lysate were performed as described in the previously mentioned study. The following antibodies were used: anti NDR1 (1:500), p-NDR1, YAP, p-YAP, MST1, LATS1, cleaved poly ADP-ribose polymerase (PARP) and β-actin (all 1:1000). For apoptosis research, tumor necrosis factor-α (TNF-α, PEPRO TECH company, USA, Catalog#: 300-01A, 10 ng/mL) was used.

CCK-8 cell proliferation assay

U87 MG and U251 cells infected with Lv-OE-NDR1 or Lv-Con lentivirus were seeded in 96-well plates at a density of 2000 cells/well and incubated in a 37 °C culture chamber for 1 day, 2 days, 3 days, 4 days, or 5 days. Then, the CCK-8 solution (Dojindo, Japan) was added to the culture medium, and cells infected with the virus were incubated with the above medium for 2 h at 37 °C in 95% humidified air and 5% CO_2. The optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad Laboratories Inc, Hercules, CA, USA). Each group had five duplicates and the experiment was performed in triplicate.

Colony formation assay

Cells infected with Lv-OE-NDR1 or Lv-Con lentivirus were seeded in 10 cm dishes (U87: 800/well; U251: 1000/

well, respectively). The supernatant was discarded and the culture medium was replaced every 3 days. After culturing for 14 days, cells were washed two times with PBS and fixed in 4% paraformaldehyde for 30 min. Finally, after washing with PBS again, the colonies were stained with 5% crystal violet for 30 min. The number of colonies (>50 cells/colony) were quantified using ImageJ (National Institutes of Health, USA). Each group had three replicates and the experiment was performed three times.

Cell cycle analysis

Infected U87 MG and U251 cells were seeded in 6-well dishes, collected by trypsinization and fixed in 75% cold ethanol for 24 h at 4 °C. After washing two times with cold PBS, cells were resuspended in 500 μL RNaseA (KeyGEN Bio Tech Company, Jiangsu, China) at 37 °C for 30 to 60 min. Then, cells were stained with 500 μL propidium iodide (KeyGEN Bio Tech company, Jiangsu, China) at room temperature for 60 min in the dark. Cell cycle distribution was determined using a flow cytometer (BD Bioscience, USA). The experiment was performed in triplicate according to the manufacture's instruction.

In vivo xenograft assay

Female nude mice were purchased from the Model Animal Research Center of Peking University Health and Science Center and housed in specific pathogen-free barrier facilities. Twenty four 6-week-old female nude mice were randomly divided into two groups. A total of 1×10^7 U87 cells infected with Lv-OE-NDR1 were injected into the right axillary of 12 mice, and the other 12 mice were injected with U87 cells infected with Lv-Con at equal concentrations. The diameters of xenografts were measured using a slide caliper every 3 days for 32 days. Xenograft tumor volume was calculated using the formula: $V = (\text{length} \times \text{width}^2)/2$. At the end of the experiment, mice were euthanized and imaged. Xenograft tumors were removed from each mouse, imaged, weighed, and fixed in 4% paraformaldehyde.

IHC staining

Nude mouse xenograft tumors were paraffin-embedded and sectioned, and then these tumor tissues were subjected to IHC staining, as previously described. Anti-NDR1 primary antibody (1:50, Santa Cruze, USA), Ki-67 antibody (1:200, Cell Signaling Technology, USA) and hematoxylin-eosin (HE) staining were performed. Patient tumors acquired from surgery were embedded and sectioned. Anti-NDR1 primary antibody (1:50, Santa Cruze, USA) and anti-YAP antibody (1:100, Abcam, USA) were used.

Immunofluorescence staining

Infected U87 MG and U251 cells were seeded in confocal dishes. After washing with PBS three times, cells were resuspended in 500 μL formaldehyde at room temperature for 5 min. Then, cells were blocked in 2% goat serum at room temperature for 30 min and incubated with anti-NDR1 primary antibody (1:100, Santa Cruze, USA), anti-YAP antibody (1:200, Abcam, USA) and DAPI (4'6-Diamidino-2-Phenylindole). Okadaic acid (OA, Cas: 78111-17-8, APEXBIO company, Houston, USA, dose: 10 nmmol/L) was used to improve the kinase activity of p-NDR1.

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay (TUNEL)

Xenograft tumors were fixed in 4% paraformaldehyde for 20 min at room temperature and rinsed with PBS. Next, slides were incubated in 0.10% Triton X-100 solution for 2 min on ice and rinsed twice with PBS. Next, 50 μL TUNEL reaction mixture was added to slides and incubated in a humidified atmosphere for 1 h at 37 °C in dark. Next, cells were rinsed with PBS and stained with Hoechst 33258. Finally, cells were analyzed under a fluorescence microscope.

Statistical analysis

Data are presented as mean ± standard deviation from at least three calculations. The chi-square test was employed to statistically compare low NDR1 expression patients and high NDR1 expression patients. For comparison of treatment groups, we performed unpaired t-tests (Mann–Whitney), or one-way or two-way ANOVA (Analysis of Variance, where appropriate). In addition, Kaplan–Meier analysis was employed to evaluate correlation between the prognosis of GBM patients and NDR1 mRNA levels in our sample. The Statistical Package for Social Science version 21 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software Inc, Sam Diego, CA, USA) were used to calculate statistics and $P < 0.0500$ was considered statistically significant.

Results

NDR1 is downregulated in GBM tissues and is negatively associated with clinical outcomes

To investigate the role of NDR1 in GBM, we measured its expression level in GBM tissues and normal tissues obtained from the pathology department. Expression levels of NDR1 were relatively lower in GBM tumor tissues than those in normal tissues [Figure 1A and 1B]. Meanwhile, we gathered general information on the patients including age, sex, tumor size, tumor extent, type of surgery, and radiation therapy [Table 1]. Analysis suggested that expression levels of NDR1 were decreased in large tumors compared to small tumors, indicating that NDR1 is negatively correlated with tumor size [Table 2]. Those patients were followed up for 15.78 ± 2.34 (13–17) months. Survival analysis also suggested that patients with lower NDR1 levels exhibited shorter overall survival times [Figure 1C]. Moreover, data from Oncomine revealed that NDR1 levels were lower in GBM tissues than those in normal tissues [Figure 1D–F]. Furthermore, data from SurvExpress (Brain-Gravendeed French GBM GSE16011) showed that higher expression of NDR1 conveyed reduced risk and lower expression conveyed higher risk. Patients who had lower expression of NDR1 showed a tendency toward poor overall survival [Figure 1G, H]. Collectively,

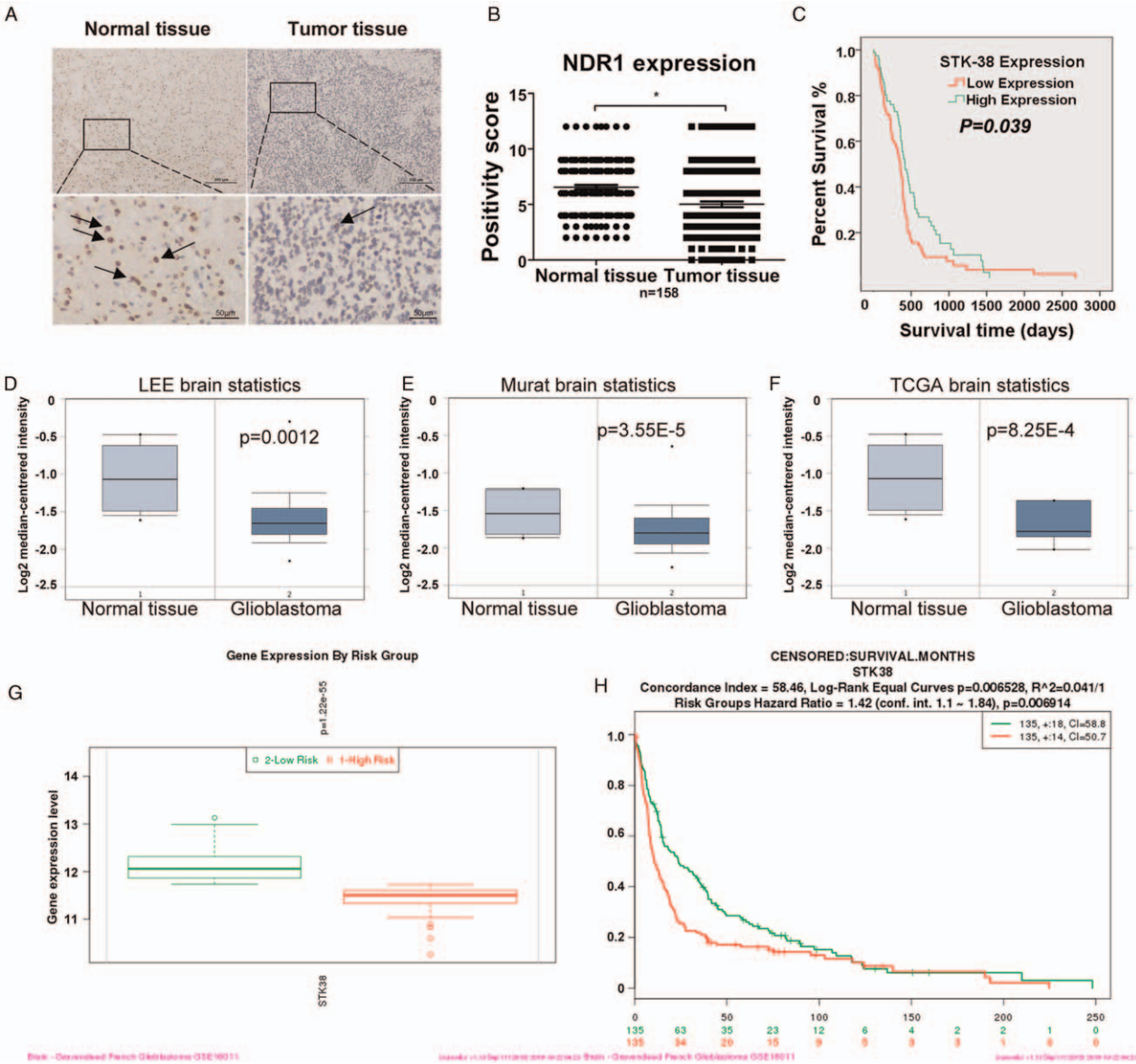

Figure 1: Expression of NDR1 is lower in GBM tissue than normal tissues. (A, B) The NDR1 protein level was lower in GBM tissue, as shown by immunohistochemical analysis (Black arrow, IHC, original magnification × 200, $n = 158$, $^{*}P < 0.0500$). (C) Association between the NDR1 level and overall survival ($n = 158$, $P = 0.0390$). (D–F) Low mRNA expression of NDR1 in GBM tissues LEE brain statistics ($P = 0.0012$), Murat brain statistics ($P = 3.55E–5.00$) and TCGA (The Cancer Genome Atlas) brain statistics ($P = 8.25E–4.00$) (ONCOMINE database). (G) Gene expression of NDR1 divided by risk (SurvExpress dataset) ($P = 1.22E–55.00$). (H) Censored survival months of NDR1 (SurvExpress dataset) ($P = 0.0069$). GBM: Glioblastoma; IHC: Immunohistochemistry; NDR1: Nuclear Dbf2-related 1.

these results indicate that NDR1 expression levels tend to be low in GBM patients, and lower expression of NDR1 is associated with poor clinical prognosis and bigger tumor size.

NDR1 decreased cell proliferation in vitro

Cell proliferation is an important indicator for evaluating cellular activity, metabolism, and physiological and pathological conditions. Based on the above findings, we next investigated the tumor suppressor effect of NDR1 in GBM. We successfully transfected U87 and U251 cells with lentivirus-mediated OE-RNA and ctrl-RNA. As is shown in Figure 2, CCK-8 results showed that NDR1-OE in both U87 and U251 cells significantly decreased tumor cell viability [Figure 2A and 2B]. After 14 days of incubation, the number of clones of NDR1 overexpressing cells was significantly less than that of NDR1-control cells [Figure 2C–F]. EdU (5-ethynyl-2′-deoxyuridine) is widely used in cell proliferation, cell differentiation, growth, and development. DNA replication activity can be directly and accurately detected based on the specific reaction between Apollo® fluorescent dye and EdU. Our Edu fluorescent images revealed that the U87-control group had a more active proliferation ability [Figure 2G–J]. In addition, cell cycle analysis suggested that U87 overexpressing group were dramatically arrested in G1 phase compared to the control, and the number of cells in the S and G2 phases was decreased. Similar results were found in U251 cells [Figure 2K–M]. Western blotting was used to further confirm cell cycle arrest and showed that cyclin-E1 was downregulated in the OE group [Figure 2N, O]. Cell

Table 1: General information of 158 patients with glioblastoma collected in this study.

Variables	Values
Age (years)	59.33 ± 13.67
Sex	
Female	56 (35.40)
Male	102 (64.60)
Tumor size (mm)	
≤20	20 (12.70)
21–40	57 (36.10)
41–60	56 (35.40)
>60	25 (15.80)
Tumor extension	
Localized at one side, not cross the mid line	131 (82.90)
Invasive, cross the mid line and distal metastasis	27 (17.10)
Type of surgery	
Partial resection	32 (20.30)
Gross resection	60 (38.00)
Radical resection	66 (41.80)
Radiation therapy	
Yes	123 (77.80)
No	22 (13.90)
Unknown	13 (8.30)

Data are presented as *n* (%) or mean ± standard deviation.

migration and invasion were also assessed [Supplementary Digital Content, Figure 2A and 2B, http://links.lww.com/CM9/A697]. The results showed that NDR1 had minimal effects on migration and invasion. Next, the Epithelial-Mesenchymal transition (EMT) pathway was detected by western blotting. The results demonstrated that NDR1 had little effect on the EMT pathway [Supplementary Digital Content, Figure 2C, http://links.lww.com/CM9/A697].

NDR1 inhibits tumor growth in vivo

To further validate the function of NDR1 in GBM tumorigenesis *in vivo*, U87 cells infected with Lv-OE-NDR1 or LV-Control were subcutaneously injected into the six-week-old nude mice and tumor sizes and weights were measured after mice were euthanized. As shown in Figure 3, the tumor size and weight of nude mice injected with Lv-OE-NDR1 infected cells were significantly lower than those of mice injected with control cells [Figure 3A, B]. The xenografts were subjected to HE staining to demonstrate that they were GBM tumors that originated from U87 cells. Furthermore, IHC results indicated that expression of NDR1 in OE tumors was higher than that in control tumors [Figure 3C and 3H]. Expression of Ki-67 in OE tumors was also lower than that in control tumors [Figure 3D and 3I]. In addition, TUNEL images showed

Table 2: Correlation between expression of NDR1 and clinical variables of GBM patients.

Variables	Expression of NDR1			
	Low (*n*)	High (*n*)	*P*	χ^2
Age (years)			0.7410	1.250
≤50	21	18		
51–60	23	19		
61–70	19	23		
>70	16	19		
Sex			0.5060	0.443
Female	30	26		
Male	49	53		
Tumor size (mm)			0.0210	9.773
≤20	8	12		
21–40	21	36		
41–60	33	23		
>60	17	8		
Tumor extension			0.8330	0.045
Localized at one side, not cross the midline	65	66		
Invasive, cross the midline and distal metastasis	14	13		
Type of surgery			0.6820	0.767
Partial resection	18	14		
Gross resection	28	32		
Radical resection	33	33		
Radiation therapy			0.0600	5.636
Yes	58	65		
No	16	6		
Unknown	5	8		

To clarify the expression of NDR1 in GBM tissues, this study collected pathological sections of tumor samples of GBM and performed immunohistochemical staining of NDR1. It was scored according to its staining strength: negative (0 points); weakly positive (1 point); positive (2 points); strong positive (3 points).[Supplementary Figure 1A]. It was scored according to the number of positive cells stained by NDR1 IHC: <5% (0 points); 10–25% (1 point); 26–50% (2 points); 50–75% (3 points), >75% (4 points) [Supplementary Figure 1B]. Final score = number of positive cells score × staining intensity score. According to the expression scores of NDR1 in GBM tissues, they were divided into low expression group (final score ≤3 points) and high expression group (final score >3 points). Survival analysis was conducted between the two groups based on the clinical prognosis of the patients. GBM: Glioblastoma; IHC: Immunohistochemistry; NDR1: Nuclear Dbf2-related 1.

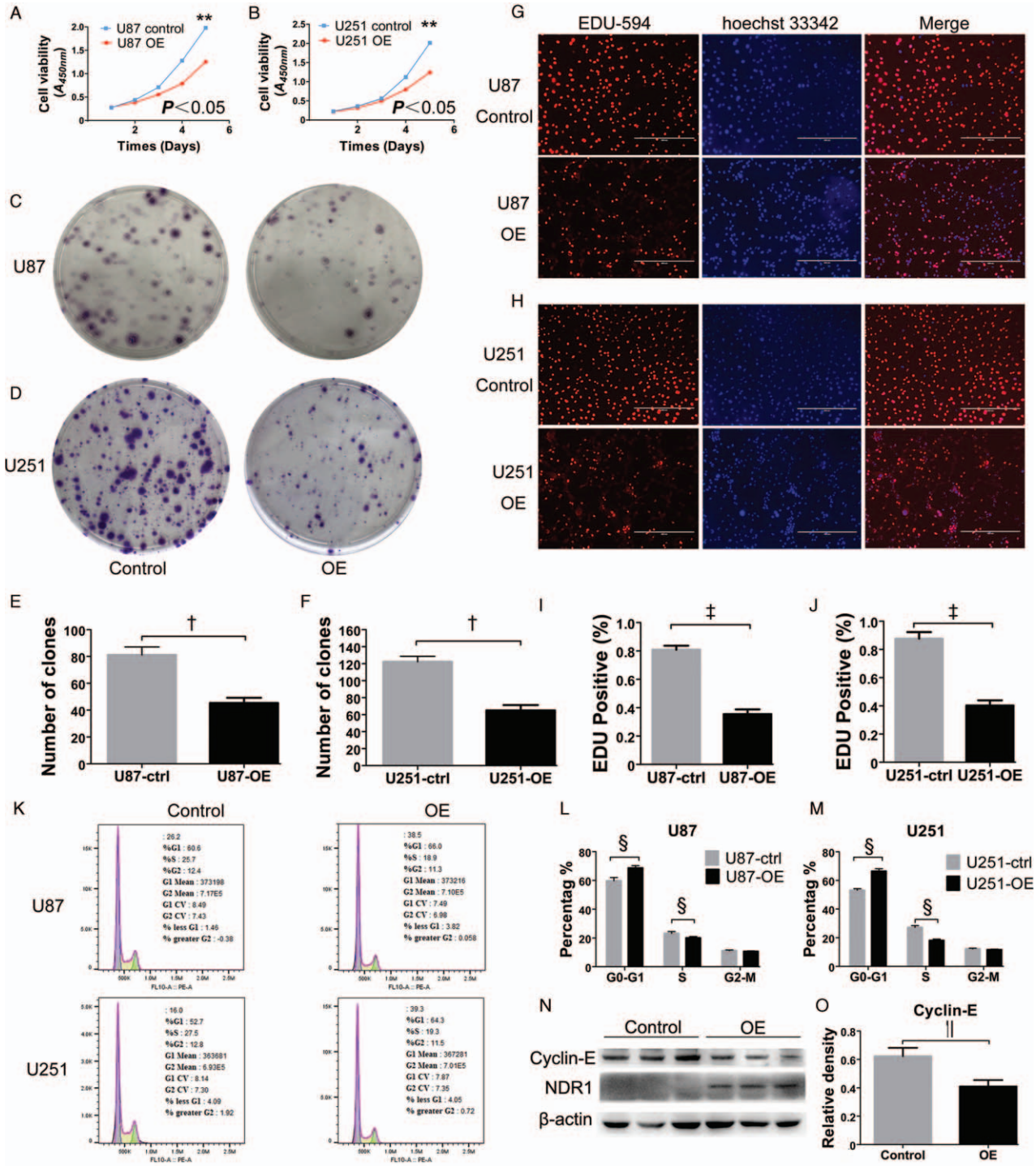

Figure 2: NDR1-OE suppressed cell proliferation *in vitro*. (A, B) Cell viability of U87 and U251 control group and OE group were validated by CCK-8 assay, $^{**}P < 0.0500$. (C–F) Number of cell clones of U87 and U251 of control group and OE group, $^{\dagger}P < 0.0500$. (G–J) EDU immunofluorescence images of U87-ctrl and U87-OE. In NDR1-OE group, the number of EDU positive cells was reduced when compared to NDR1-ctrl group (Original magnification x100, $^{\ddagger}P < 0.0500$). (K–M) cell cycle analysis result of U87-ctrl and U87-OE groups, U251-ctrl and U251-OE groups. NDR1 overexpression significantly arrested cell cycle at G1 phase with a concomitant reduction in proportion of S phase ($^{\S}P < 0.0500$). (N, O) Western blotting results of cyclin-E1 and NDR1. In NDR1-OE group, the expression of cyclin-E1 was reduced when compared to NDR1-ctrl group ($||P < 0.0500$). CCK-8: Cell counting kit-8; CON: control group; EDU: 5-ethynyl-2′-deoxyuridine; NDR1: Nuclear Dbf2-related 1; OE: Overexpression group.

that NDR1 overexpressing promoted apoptosis [Figure 3E and 3J]. Both the tumor volume and tumor weight were significantly reduced in the OE group [Figure 3F and 3G]. These results demonstrate that NDR1 upregulation inhibits the tumorigenicity of GBM cells *in vivo*.

NDR1 combines with and phosphorylates YAP

Considering that NDR1 and LATS belong to the same AGC family, we hypothesized that NDR1 might also interact with YAP. To investigate the interaction between NDR1 and YAP, immunofluorescence was performed on

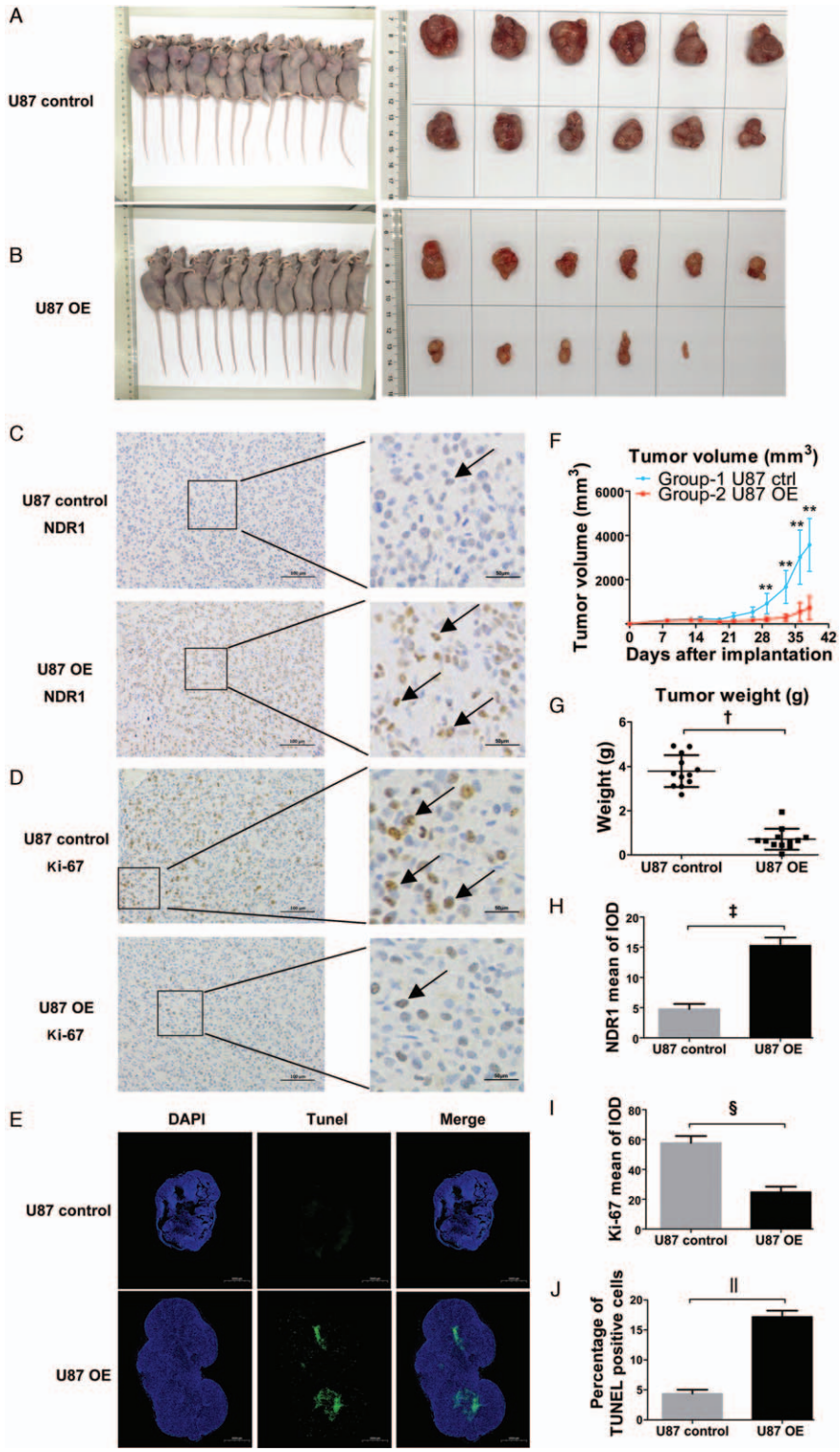

Figure 3: NDR1-OE inhibited tumor growth *in vivo*. (A, B) Representative picture of animals and xenograft tumors. (C) Representative expression of NDR1 of xenograft from U87-ctrl and U87-OE group. (Black arrow, IHC, original magnification × 200) (D) Representative expression of Ki-67 from U87-ctrl and U87-OE group. (Black arrow, IHC, original magnification × 200) (E) Terminal Deoxynucleotidyl Transferase Deoxyuridine TUNEL images of U87-ctrl and U87-OE. (Original magnification x40) (F–G) statistical results of tumor volume ($^{**}P < 0.0500$) and tumor weight statistical results ($^{\dagger}P < 0.0500$). (H–J) Statistical results of NDR1 ($^{\ddagger}P < 0.0500$), Ki-67 ($^{\S}P < 0.0500$) and TUNEL staining ($||P < 0.0500$). CON: control group; IHC: Immunohistochemistry; IOD: integrated optical density; NDR1: Nuclear Dbf2-related 1; OE: Overexpression group; TUNEL: Triphosphate Nick-End Labeling Assay; YAP1: Yes-associated protein 1.

it. U87 cells were transfected with NDR1-OE plasmids with mscarlet. Immunofluorescence was performed when mscarlet was observed under microscope. Immunofluorescence microscopy revealed that NDR1 and YAP co-localize in human GBM cells [Figure 4A; Supplementary Digital Content, Figure 3, http://links.lww.com/CM9/

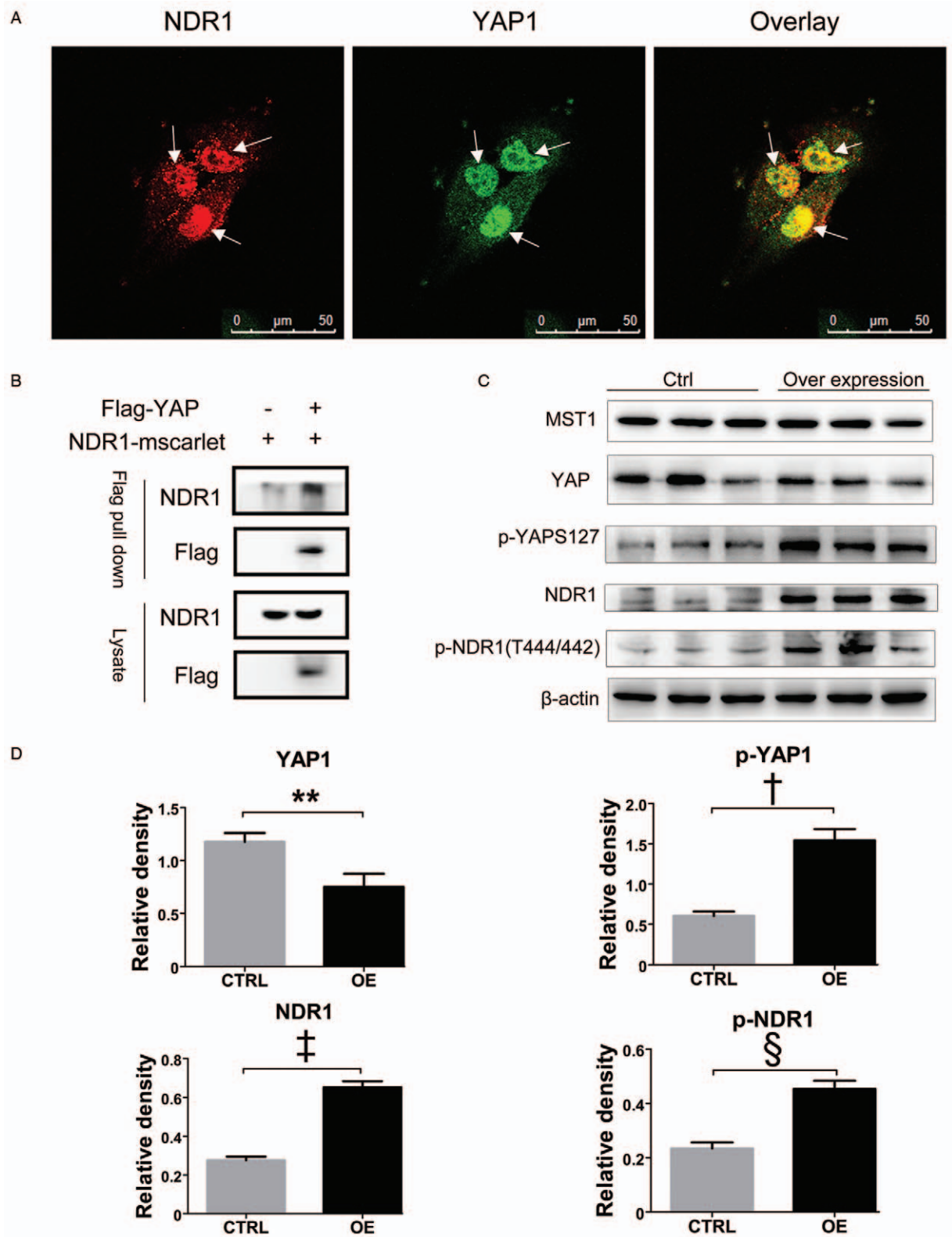

Figure 4: NDR1 phosphorylate YAP on Ser127. (A) Immunofluorescence with confocal microscopy images of NDR1 and YAP. The overlap image showed that NDR1 significantly colocalizes with YAP. (White arrow, colocalization of NDR1 and YAP, immunofluorescence, Original magnification × 630) (B) Co-IP results of NDR1 and YAP. Results showed that NDR1 could interact with YAP. (C) Endogenous protein levels of total YAP, phospho-127 YAP, MST1, NDR1 and phosphorylated NDR1 (T444/442) and β-actin were examined by western blotting. (D) Statistical results of total YAP ($^{**}P < 0.0500$), phospho-127 YAP ($^{\dagger}P < 0.0500$), NDR1 ($^{\ddagger}P < 0.0500$) and p-NDR1 (T444/442) ($^{\S}P < 0.0500$). Co-IP: CO-immunoprecipitation; Ctrl: control group; MST1/2: Mammalian sterile-20-like 1/2; MST1: Mammalian sterile-20-like 1; NDR1: Nuclear Dbf2-related 1; OE: Overexpression group; p-YAP: phosphorylated YAP; YAP: Yes-associated protein.

A698]. To further verify the interaction between NDR1 and YAP, co-IP was performed. We co-transfected NDR1-mscarlet and Flag-tagged YAP into U87 cells. Immunoprecipitation using an anti-Flag antibody revealed that NDR1 was coimmunoprecipitated with YAP. To rule out OE artifacts, we examined the interaction between endogenous NDR1 and YAP in lysates, showing that YAP coimmunoprecipitated NDR1 [Figure 4B]. This result identified that NDR1, in addition to LATS1/2, interacts with YAP. Posttranslational modifications including phosphorylation and acetylation are critical for the protein stability and functional activity of YAP. Therefore, we explored whether upregulated NDR1 increases YAP stability by modulating YAP phosphorylation. We found that NDR1 phosphorylates YAP at S127 site [Figure 4C and 4D].

NDR1 regulates the localization and transcriptional activity of YAP

Considering that phosphorylation of YAP can result in the inactivation of YAP by cytoplasmic retention, we hypothesized that NDR1 OE might reduce nuclear YAP1 levels. To test this hypothesis, we performed immunofluorescence staining on it. Phosphorylation of YAP is thought to promote YAP cytoplasmic localization and inactivation. OA is a kind of phosphatase inhibitor. Studies have shown that OA causes a global increase in phosphorylation levels including levels of p-NDR1. When OA was added to U87 cells, more YAP localized in the cytoplasm in the NDR1-OE group than in the control group [Figure 5A]. The ratio of nuclear *vs.* cytoplasmic YAP1 was significantly reduced in cells overexpressing NDR1 compared to controls [Figure 5B].

NDR1 could mediate apoptosis of TNF-α stimulation

Because the TUNEL staining results suggested that the degree of apoptosis was significantly higher in the NDR1-OE group than that in the NDR1-control group, we performed further research on the role of NDR1 in apoptosis in U87 cells by western blotting. As shown in Figure 6, after TNF-α stimulation for 4 h, expression of cleaved PARP was higher in NDR1-OE group than that in the NDR1-control group with or without TNF-α and H_2O.

Discussion

YAP was found to be an oncogene in GBM, which is the most common type of malignant glioma with a median survival of 15 months.[1] Researchers found that YAP is highly expressed in gliomas and promotes tumor cell proliferation and invasion in glioma, indicating that regulation of YAP is critical for tumor proliferation.[13,14] YAP is the downstream core effector molecule of the Hippo pathway, which was first discovered in Drosophila and regulates organ growth, development, proliferation, and apoptosis.[6,15] A large number of studies have found that the Hippo pathway is closely related to the occurrence of many malignant tumors.[6,16] Furthermore, the Hippo pathway is abnormal in many malignant tumors, resulting in increased nonphosphorylated YAP content. As a core effector molecule, YAP is located most downstream of the

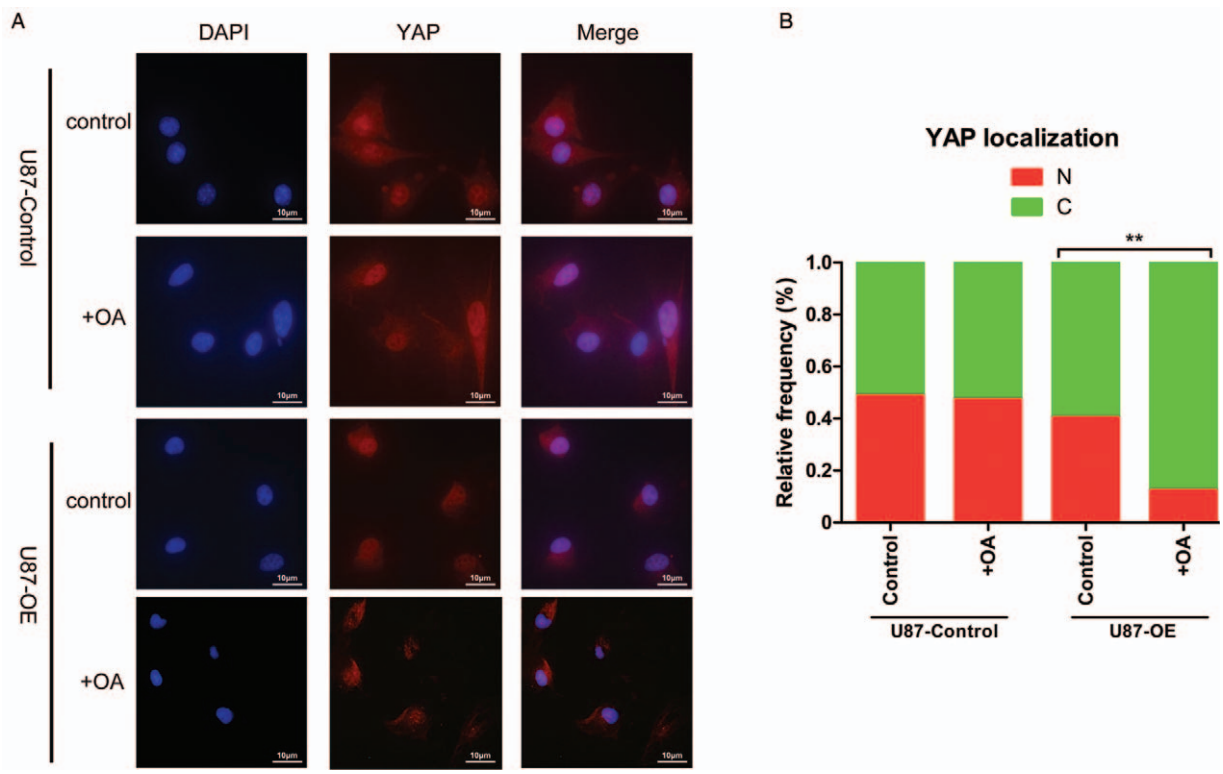

Figure 5: NDR1 regulates cellular distribution of YAP. (A) Cellular distribution of endogenous YAP in U87-ctrl and U87-OE in Okadaca acid (OA) inducible manner. When stimulated with OA, more YAP were restrained in cytoplasm. (Immunofluorescence, Original magnification x200) (B) quantification of the nuclear/cytoplasmic YAP ratio per cell. ($^{**}P < 0.0500$). C: Cytoplasm; CON: control group; N: Nucleus; NDR1: Nuclear Dbf2-related 1; OA: Okadaic acid; OE: Overexpression group; YAP: Yes-associated protein.

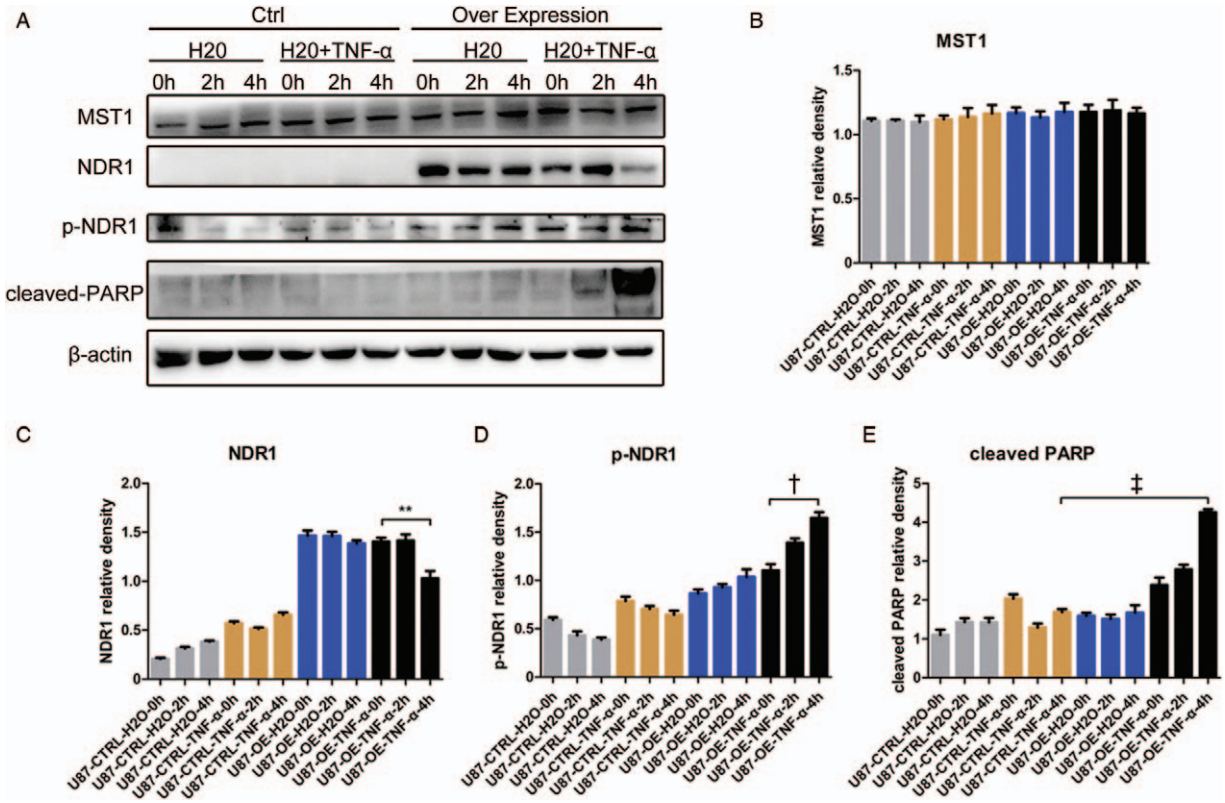

Figure 6: NDR1 mediates cell apoptosis. (A) U87-ctrl and U87-OE cells were stimulated with or without H_2O and TNF-α for 0 h, 2 h, and 4 h. The protein level of MST1, NDR1 and p-NDR1 (T444/442), cleaved PARP and β-actin were examined by western blotting. (B–E) Statistical results of NDR1 ($^{**}P < 0.0500$) and p-NDR1 (T444/442) ($^{\dagger}P < 0.0500$) and cleaved PARP ($^{\ddagger}P < 0.0500$). Ctrl: control group; MST1/2: Mammalian sterile-20-like 1/2; MST1: Mammalian sterile-20-like 1; NDR1: Nuclear Dbf2-related 1; OE: overexpression group; PARP: poly ADP-ribose polymerase; TNF-α: Tumor necrosis factor-α.

Hippo pathway, and its upstream effector molecules include MST1/2, LATS1/2 and other kinases. MST1/2 can phosphorylate LATS1/2 and LATS1/2 can phosphorylate YAP, making it inactive, retaining it in the cytoplasm, and disabling it from entering the nucleus to function.

NDR1/2 was speculated to be a tumor suppressor similar to LATS1/2 in the Hippo pathway because of their similarities. NDR and LATS belong to the NDR/LATS family and both of them need to be phosphorylated on two residues to achieve full kinase activation, similar to other AGC kinases.[17] Notably, all NDR/LATS kinases share two unique characteristics including a conserved N-terminal regulatory domain (NTR) in proximity to the catalytic domain and insertion between subdomains VII and VIII of the catalytic domain.[18] Both of these two domains play roles in the regulation of NDR/LATS kinases. A previous study also showed that the NDR/LATS family, including NDR, LATS, Orb6 and Cot-1, has the same sequences of phosphorylation sites corresponding to both Ser-281 and Thr-444, which means that the NDR/LATS family is highly conserved.[19] NDR1/2 plays its role through the phosphorylation of two sites, Ser-281 and Thr-444. Therefore, it is speculated that NDR/LATS family might have similar functions. Studies have also shown that Ste20-like Hippo kinases activate the fly NDR/LATS kinase Warts.[20] Specifically, NDR1/2 can be phosphorylated by MST1/2 on its Thr residue located in HM and is regulated by MOB1. In this regard, NDR1/2 could be considered a novel member of Hippo pathway. Consequently, we speculate that NDR1/2 may represent a tumor suppressor.

Many studies have shown that NDR1/2 exert a tumor suppressor effect. Cui *et al*[21] found that the expression of NDR1 in cancer tissues of patients with gastric cancer was significantly lower than that of normal gastric mucosa through gene chip research. They proposed that the expression levels of NDR1 can be used for early warning of gastric cancer. In a chemically induced mouse skin cancer model, Hummerich *et al*[22] used gene expression analysis and found that compared to normal skin tissue, expression of NDR2 in skin tumor specimens was significantly reduced. The authors also performed immunofluorescence to verify the result, which was indeed the case. Finally, using q-PCR analysis, cancer-promoting factors were shown to be significantly increased, while the expression of NDR2 was significantly reduced. Messina *et al*[23] conducted an oligonucleotide array to study the *PK* gene expression profile in adult acute lymphoblastic leukemia (ALL) samples and found that expression of NDR1 was significantly reduced in ALL. A recent study by Zhang *et al*[24] found that intestinal epithelial cells from NDR1 null mice with conditional knockout of NDR1/2 were exposed to azoxymethane/dextran sodium sulfate enema to observe whether mice experienced resultant colon cancer. The results showed that there were fewer nodules in the colon of control mice, but greater than ten nodules in

the colon of NDR1/2 cKO mice, indicating that NDR1/2 exerts an antitumor effect.

However, NDR has also been found to be elevated in some tumors. Adeyinka *et al*[25] found that compared to ductal carcinoma *in situ* (DCIS) without necrosis, mRNA expression levels of NDR1 were higher in DCIS with necrosis, but did not reach a statistically significant difference. In addition, the high expression of NDR1 is closely related to the proliferation of B-cell lymphoma.[26] Bisikirska *et al*[26] found that in human B-cell lymphoma, NDR1 affects the stability of MYC protein in a kinase activity-dependent manner. When NDR1 is silenced, the stability of MYC protein and the survival rate of lymphoma cells are significantly reduced. NDR1 also regulates the transcriptional activity of MYC. Moreover, to evaluate the effect of NDR1 knockdown on tumorigenic growth of MYC-dependent human lymphomas *in vivo*, the authors introduced xenografts of stable NDR1 shRNA cell lines into SCID mice and found that tumor growth was delayed by 8 days, indicating that NDR1 knockdown inhibits tumor growth. Taken together, NDR1 is a versatile molecule that plays a differential role in the progression of different cancers. However, its role in glioma remains unknown. This study suggested that NDR1 inhibits tumor proliferation ability but has little effect on migration or invasion.

As the upstream regulator of YAP, LATS1/2 is considered a tumor suppressor gene. For a long time, LATS kinase has been established as the only regulator of YAP and TAZ.[27,28] There is no evidence that NDR1/2 also mediates the phosphorylation of YAP.[6,24] 10 years ago, Hao *et al*[29] found that NDR1/2 does not interact with YAP. Therefore, he believed that NDR1/2 is not a kinase that affects YAP in mammals. However, Hao *et al* did not perform additional in-depth research on NDR1/2. Other studies have shown that a decrease in NDR1/2 levels is related to an increase in TAZ activity, but the direct link between NDR1/2 and TAZ has not yet been established.[17] In recent years, Zhang *et al*[24] postulated a new conclusion. They used azoxymethane/Portugal Glycan sodium sulfate enema to stimulate intestinal epithelial cells of Ndr1 null mice with conditional knockout of NDR1/2 to investigate whether it induced colon cancer. Results showed that there were fewer nodules in the colons of normal control mice, but more than 10 nodules in the colon of NDR1/2 cKO mice, indicating that NDR1/2 has an antitumor effect. Furthermore, the author found that after knocking out NDR1/2, the expression level of nonphosphorylated YAP was significantly increased, and the expression level of p-YAP was significantly reduced, indicating that NDR can have an anti-tumor proliferation effect through phosphorylation of YAP. His conclusion was summarized from mice. We examined whether the NDR1/2-YAP interaction exists in humans. This study suggests that NDR1 phosphorylates YAP at S127 and plays a role in mammalian cells. Moreover, our study found that NDR1 directly interacts with YAP to play its role, which conflicts with Hao's findings. Our results suggest that LATS is not the only upstream regulator of YAP, which is a significant supplement to our understanding of the Hippo pathway.

In addition, NDR1 also mediates the apoptotic process to act as a tumor suppressor. Apoptosis is an important mechanism of maintaining homeostasis and inhibiting tumor formation. In cancer cells, apoptosis is often suppressed by the inactivation of corresponding tumor suppressor factors.[30] It has been reported that reintroduction of RASSF1A induces cell cycle inhibition by inactivating the APC/Cdc20 complex.[31] Studies have shown that Fas and TNF-α receptors are stimulated by promoting the phosphorylation of the NDR1/2 hydrophobic motif (Thr444/442). In addition, NDR1/2 is necessary for Fas receptor-induced apoptosis. Fas receptor stimulation promotes the direct phosphorylation of MST1 and activation of NDR1/2. In addition, the apoptosis induced by RASSF1A largely depends on the presence of NDR1/2.[32] Cornils *et al*[33] found that in NDR1-deficient mice, expression of NDR1 can be activated after endogenous and exogenous pro-apoptotic stimuli are administered, indicating that NDR1 plays an important role in regulating cell apoptosis.

Since YAP is known as a biomarker of GBM, it is beneficial to investigate the question of what molecules can phosphorylate YAP. Our data suggest that NDR1 inhibits GBM progression by phosphorylating YAP. We found that YAP binds to NDR1 and is phosphorylated into p-YAP at S127. In addition, when NDR1 was overexpressed, greater levels nonphosphorylated YAP were localized in the cytoplasm. The effect of NDR1 on phosphorylated YAP provides us with a potential novel target for glioma therapy. This discovery is very important for the understanding of the Hippo pathway, which determined that NDR1/2 can be treated as an important supplement to the Hippo pathway. Our study also indicates that the Hippo pathway does not phosphorylate YAP simply through LATS. In fact, NDR1 may even have a more important role than LATS1/2.

Studies found that some independent risk factors that affect the prognosis of GBM, including radiotherapy, which means that radiotherapy has a positive effect on improving the prognosis of GBM. However, the effect of NDR1 on radiotherapy is unclear. Temozolomide, as the only effective drug for the treatment of GBM, is widely used clinically. It is unclear whether NDR1 can enhance or reduce the efficacy of temozolomide. Our research object in this study is mainly for GBM (WHO grade IV), but according to the latest molecular classification, the expression level of NDR1 in different types of GBM is still unclear.

In summary, our findings indicate that NDR1 functions as a tumor suppressor in GBM. NDR1 was identified as a novel regulator of YAP, which provides a new in-depth comprehension in Hippo signaling pathway.

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Conflicts of interest

None.

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. Neuro Oncol 2014;16:896–913. doi: 10.1093/neuonc/nou087.
2. Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, *et al*. Cilengitide combined with standard treatment for patients with newly diagnosed GBM with methylated MGMT promoter (CENTRIC EORTC 26071-22072 study): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol 2014;15:1100–1108. doi: 10.1016/S1470-2045(14)70379-1.
3. Duan H, Hu JL, Chen ZH, Li JH, He ZQ, Wang ZN, *et al*. Assessment of circulating tumor DNA in cerebrospinal fluid by whole exome sequencing to detect genomic alterations of GBM. Chin Med J (Engl) 2020;133:1415–1421. doi: 10.1097/CM9.0000000000000843.
4. Zhao B, Li L, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: An updated version. Genes Dev 2010;24:862–874. doi: 10.1101/gad.1909210.
5. Hong W, Guan KL. The YAP and TAZ transcription co-activators: Key downstream effectors of the mammalian Hippo pathway. Semin Cell Dev Biol 2012;23:785–793. doi: 10.1016/j.semcdb.2012. 05.004.
6. Yu FX, Zhao B, Guan KL. Hippo pathway in organ size control, tissue homeostasis, and cancer. Cell 2015;163:811–828. doi: 10.1016/j.cell.2015.10.044.
7. Orr BA, Bai H, Odia Y, Jain D, Anders RA, Eberhart CG. Yes-associated protein 1 is widely expressed in human brain tumors and promotes GBM growth. J Neuropathol Exp Neurol 2011;70:568–577. doi: 10.1097/NEN.0b013e31821ff8d8.
8. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, *et al*. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev 2007;21:2747–2761. doi: 10.1101/gad.1602907.
9. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002;298:1912–1934. doi: 10.1126/science.1075762.
10. Hergovich A, Stegert MR, Schmitz D, Hemmings BA. NDR kinases regulate essential cell processes from yeast to humans. Nat Rev Mol Cell Biol 2006;7:253–264. doi: 10.1038/nrm1891.
11. Geng W, He B, Wang M, Adler PN. The tricornered gene, which is required for the integrity of epidermal cell extensions, encodes the Drosophila nuclear Dbf2-related kinase. Genetics 2000;156:1817–1828.
12. Zallen JA, Peckol EL, Tobin DM, Bargmann CI. Neuronal cell shape and neurite initiation are regulated by the Ndr kinase SAX-1, a member of the Orb6/COT-1/warts serine/threonine kinase family. Mol Biol Cell 2000;11:3177–3190. doi: 10.1091/mbc.11.9.3177.
13. Wang Y, Pan P, Wang Z, Zhang Y, Xie P, Geng D, *et al*. β-catenin-mediated YAP signaling promotes human glioma growth. J Exp Clin Cancer Res 2017;36:136. doi: 10.1186/s13046-017-0606-1.
14. Zhang Y, Xie P, Wang X, Pan P, Wang Y, Zhang H, *et al*. YAP promotes migration and invasion of human glioma cells. J Mol Neurosci 2018;64:262–272. doi: 10.1007/s12031-017-1018-6.
15. Huang J, Wu S, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell 2005;122:421–434. doi: 10.1016/j.cell.2005.06.007.
16. Pan D. The Hippo signaling pathway in development and cancer. Dev Cell 2010;19:491–505. doi: 10.1016/j.devcel.2010.09.011.
17. Hergovich A. The roles of NDR protein kinases in Hippo signalling. Genes (Basel) 2016;7:21. doi: 10.3390/genes7050021.
18. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol 2010;11:9–22. doi: 10.1038/nrm2822.
19. Millward TA, Hess D, Hemmings BA. Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. J Biol Chem 1999;274:33847–33850. doi: 10.1074/jbc.274.48.33847.
20. Wu S, Huang J, Dong J, Pan D. Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 2003;114:445–456. doi: 10.1016/s0092-8674(03)00549-x.
21. Cui DX, Zhang L, Yan XJ, Zhang LX, Xu JR, Guo YH, *et al*. A microarray-based gastric carcinoma prewarning system. World J Gastroenterol 2005;11:1273–1282. doi: 10.3748/wjg.v11.i9.1273.
22. Hummerich L, Müller R, Hess J, Kokocinski F, Hahn M, Fürstenberger G, *et al*. Identification of novel tumour-associated genes differentially expressed in the process of squamous cell cancer development. Oncogene 2006;25:111–121. doi: 10.1038/sj.onc.1209016.
23. Messina M, Chiaretti S, Tavolaro S, Peragine N, Vitale A, Elia L, *et al*. Protein kinase gene expression profiling and in vitro functional experiments identify novel potential therapeutic targets in adult acute lymphoblastic leukemia. Cancer 2010;116:3426–3437. doi: 10.1002/cncr.25113.
24. Zhang L, Tang F, Terracciano L, Hynx D, Kohler R, Bichet S, *et al*. NDR functions as a physiological YAP kinase in the intestinal epithelium. Curr Biol 2015;25:296–305. doi: 10.1016/j.cub.2014.11.054.
25. Adeyinka A, Emberley E, Niu Y, Snell L, Murphy LC, Sowter H, *et al*. Analysis of gene expression in ductal carcinoma in situ of the breast. Clin Cancer Res 2002;8:3788–3795.
26. Bisikirska BC, Adam SJ, Alvarez MJ, Rajbhandari P, Cox R, Lefebvre C, *et al*. STK38 is a critical upstream regulator of MYC's oncogenic activity in human B-cell lymphoma. Oncogene 2013;32:5283–5291. doi: 10.1038/onc.2012.543.
27. Oh H, Irvine KD. Yorkie: The final destination of Hippo signaling. Trends Cell Biol 2010;20:410–417. doi: 10.1016/j.tcb.2010.04.005.
28. Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. Genes Dev 2016;30:1–17. doi: 10.1101/gad.274027.115.
29. Hao Y, Chun A, Cheung K, Rashidi B, Yang X. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J Biol Chem 2008;283:5496–5509. doi: 10.1074/jbc.M709037200.
30. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. Oncogene 2004;23:2950–2966. doi: 10.1038/sj.onc.1207558.
31. Song MS, Song SJ, Ayad NG, Chang JS, Lee JH, Hong HK, *et al*. The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. Nat Cell Biol 2004;6:129–137. doi: 10.1038/ncb1091.
32. Vichalkovski A, Gresko E, Cornils H, Hergovich A, Schmitz D, Hemmings BA. NDR kinase is activated by RASSF1A/MST1 in response to Fas receptor stimulation and promotes apoptosis. Curr Biol 2008;18:1889–1895. doi: 10.1016/j.cub.2008.10.060.
33. Cornils H, Stegert MR, Hergovich A, Hynx D, Schmitz D, Dirnhofer S, Hemmings BA. Ablation of the kinase NDR1 predisposes mice to the development of T cell lymphoma. Sci Signal 2010;3:ra47. doi: 10.1126/scisignal.2000681.

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