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The tumor suppressor NOR1 suppresses cell growth, invasiveness, and tumorigenicity in glioma

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Oxidored-nitro domain-containing protein 1 (NOR1) is a tumor suppressor downregulated in various human cancers, including nasopharyngeal carcinoma (NPC), lung cancer, and testicular cancer. NOR1 protein is highly expressed in the normal brain; however, its role in brain tumors remains unknown. In this study, we demonstrated that the NOR1 protein level was decreased in glioma tissue samples as compared to its normal counterpart. Exogenously expressed NOR1 protein in glioma U251 cells inhibits tumor cell proliferation, migration, and invasion. Re-expression of NOR1 induced cell cycle S to G2 phase arrest and suppressed its tumorigenicity in nude mice. Overexpression of NOR1 in U251 cells also led to a decrease of Ki67 expression in xenografts. Transcriptomic analysis revealed that NOR1 expression altered the expression of genes favored cell proliferation. Among the differentially expressed genes, FOXR2, a member of the FOX gene family, which promotes glioma progression, was decreased in NOR1 expressing cells. The downregulation of FOXR2 by NOR1 was validated *in vitro* and *in vivo*. Our findings suggest for the first time that NOR1 suppresses glioma progression via modulating the FOXR2 expression.

Key words: glioma, FOXR2, NOR1, tumor suppressor

Glioma is one of the most common types of primary malignancy in the central nervous system seriously threatening human health [1, 2]. Malignant glioma, a highly invasive tumor, causes an annual incidence of 3–5 per 100,000 individuals, usually in adults over 45 years of age [3, 4]. The main clinical symptom is presented as memory loss, headaches, seizures, focal neurological deficits, and even personality disruptions. Glioma can diffusely invade and infiltrate surrounding brain tissue, and intertwine each other among native cells [5]. Due to its invasive and therapy-resistant nature, most of these tumors are incurable. Generally, gliomas fall into three categories: oligodendrogliomas, astrocytomas, and ependymomas [6]. Approximately half of all newly diagnosed glioma is considered as glioblastoma, which belongs to astrocytomas and is the most malignant type of brain cancer. Despite the advance of multimodal therapeutics, the overall five-year survival rate of glioma patients, especially those with high-grade glioblastoma multiforme, is not optimistic; most of those patients only have 1-year median survival rate [7].

The oxidored-nitro domain-containing protein 1(NOR1, also called organic solute carrier partner 1, or OSCP1) is a putative tumor suppressor gene, which was downregulated in several kinds of cancers, such as nasopharynx, gastric, colon, rectum and cervical cancers. The main epigenetic reason for NOR1 silencing in these cancers may be attributed to the hypermethylation of NOR1 promoter, which has been observed in NPC and hematological malignancies [8]. NOR1 gene is located in a 120 kb region at 1p34.3 and was isolated from NPC for the first time in 2003 [9]. The protein encoded by this downregulated gene possesses a great deal of structural similarity to the nitroreductase of *Salmonella typhimurium*. NOR1 protein upregulated mitochondrial Bax/Bcl-2 ratio, thus enabling cytochrome C release to the cytoplasm and triggering apoptosis induced by hypoxia [10]. When overexpressed in *Drosophila* eye imaginal discs, NOR1 induced endoplasmic reticulum stress and apoptosis thus leading to a rough eye phenotype in adult flies [11]. Re-expression of NOR1 suppresses NPC cell growth and tumorigenicity in nude mice through inhibiting autophagy

and glycolysis [12]. In addition, to suppress cancer cell proliferation, re-expression of NOR1 in NPC cells resulted in the reversal of epithelial-mesenchymal transition and suppressed tumor invasiveness *in vitro* and metastasis *in vivo* [13–15]. Human NOR1 protein, a solute carrier protein that mediates various kinds of organic solutes in a pH-dependent and sodium-independent manner, is highly expressed in the normal brain, especially in neurons [16]. So far, the function of NOR1 in epithelial cells derived cancers has been intensively studied. However, its role in tumor development of the central nervous system is still remaining elusive.

In this study, we demonstrated NOR1 protein was decreased in glioma. Low expression of NOR1 protein predicted unfavorable clinical outcome. Overexpression of NOR1 in glioma suppressed U251 cells proliferation, migration, and invasiveness *in vitro*, and their tumorigenicity in nude mice.

Patients and methods

Tumor tissue samples from patients. A cohort of 236 subjects with glioma, as well as non-cancerous brain tissue control subjects, were recruited between July 2008 and May 2017 from the Pathology Department of the Affiliated Hospital of Jining Medical University (Shandong, China), and their tissue samples were used for immunohistochemical analysis of NOR1 expression. Clinicopathological data were also collected from patients' medical records, including gender, age, clinical stage, lymph node metastasis, and pathology diagnosis. The age of all patients ranged from 4 to 81 years (mean age of 50.56 years). 119 participants (50.42%) were males and 117 (49.58%) were females. In accordance with the WHO classification system, 6 cases were WHO I, 84 cases were WHO II, 45 cases were WHO III and 74 cases were WHO IV. This study was approved by the Institute Research Ethics Committee for the use of clinical samples and each patient signed a consent form to participate in the study.

Immunohistochemistry. NOR1 protein was measured by immunohistochemical staining as described previously [17, 18]. Briefly, immunohistochemistry assay was performed using the peroxidase-antiperoxidase technique after a microwave antigen retrieval procedure. Sections were incubated with polyclonal anti-NOR1 (#12598-1-AP, Proteintech Group, Chicago, USA), anti-Ki67 (#E607238, BBI Life Sciences, Shanghai, China) and anti-FOXR2 (#14111-1-AP, Proteintech Group, Chicago, USA) antibodies overnight at 4 °C. A staining index (values, 0–6) obtained as the staining intensity (scores: negative = 0, weak = 1, moderate = 2, or strong = 3) and % of staining of tumor cells (scores: <10% = 1, 10–50% = 2, >50% = 3) were calculated. The sum of these two scores was used as the final immunoreactive score (0–6), i.e., low expression (0–1 scores) and high expression (2–6 scores).

Cell culture and lentivirus infection. U251, a worldwide broadly used glioma cell line, was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY) in a humidified incubator at 37 °C with 5% CO_2 and 95% air. NOR1 was stably introduced into U251 cell line by using a NOR1-expressing lentivirus. Stable transfectants were selected by puromycin for 1 week. A GFP expressing lentivirus was used as control.

RNA isolation, reverse transcription and quantitative real-time PCR. Total RNA from cells was extracted using Trizol (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. The residual DNA was removed by treating with DNase I (Roche Diagnostics, Rotkreuz, Switzerland). Total RNA (3 μg) was reverse-transcribed to cDNA with M-MLV Reverse Transcriptase Kit (Thermo Fisher, Rockford, IL, USA). The resulted cDNA was used for real-time PCR with SYBR Green qPCR Master Mix (Thermo Fisher) on CFX96 Touch™ Real-Time PCR Detection System (BioRad) following the manufacturer's instruction. The thermal cycling conditions included an initial denaturation step at 95 °C for 30 s and 40 cycles of 95 °C for 5 s followed by 60 °C for 30 s. The results of three independent experiments were used to calculate the average value. The relative NOR1 mRNA levels were calculated by normalization to GAPDH mRNA levels. The PCR primers were as follows: NOR1 forward, 5'-TCAAGGGATTCATCCGAGAC-3'; NOR1 reverse, 5'-CTGGCCAAGAAATTCAGCTC-3'; FOXR2 forward, 5'-TCCCTCCAGTCCCCTGAAAT-3'; FOXR2 reverse, 5'-GCTAGGGCAATAAGGTGGCT-3'; GAPDH forward, 5'-AACGGATTTGGTCGTATTGG-3'; GAPDH reverse, 5'-TTGATTTTGGAGGGATCTCG-3'.

Protein extraction and western blot. Total cellular protein was extracted from the cultured cells using a lysis buffer (Beyotime, Jiangsu, China) and equal amount of protein samples were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Millipore, Billerica, MA). The membranes were immunoblotted with the following antibodies: polyclonal anti-NOR1 (Proteintech Group, Chicago, IL, USA), anti-FOXR2 (Proteintech Group, Inc., Chicago, IL) and anti-GAPDH antibodies (Proteintech Group, Inc., Chicago, IL).

Cell viability assay. A proliferation assay was carried out using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's protocol. U251 cells infected with NOR1 expressing lentivirus or scrambled lentivirus were seeded into 96-well plates at a density of 2×10^3 cells per well and grown for 0, 24, 48, 72 h. 10 μl CCK-8 reagent was added to each well and the cells were incubated at 37 °C for 2 h. The absorbance was recorded at 450 nm with a microplate reader (Bio-Rad, Hercules, CA). GraphPad Prism 5 was used for drawing the proliferation curves.

Colony formation assay. Approximately 2,000 cells were seeded into six-well plates in triplicate and incubated for 10–14 days. Colonies were stained with crystal violet and counted.

Wound healing assay. Cell suspensions were seeded onto a 6-well plate at a density of 8×10^5–1×10^6 cells per well. Monolayer cell cultures were scratched by using a 10 μl tip, and then the non-adhered cells were removed by washing with PBS. Cells were allowed for growth for 24 h in serum-free medium. Cell migration was observed by using a microscope.

Migration and invasion assays. Tumor cells migration and invasion assays were performed as described previously [18]. Briefly, cells were seeded onto transwell membrane inserts (8 μm, Corning) in a serum-free medium. 15% FBS containing media was added to the lower chamber. After 6 h to 24 h incubation at 37 °C, cells that migrated to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. For each membrane, 5 random fields were counted at ×10 magnification. The mean was calculated and data are presented as mean ± SEM from three independent experiments performed in triplicate. For the tumor cell invasion assay, the membrane was pre-coated with 15 μl Matrigel (BD Biosciences, Bedford, MA), and the rest of the method was identical to the tumor cell migration assay except incubation of cells was conducted at 24 to 48 h at 37 °C.

Cell cycle assay. U251 cells were washed with cold phosphate-buffered saline (PBS) and then fixed with 75% ethanol overnight at −20 °C. The fixed cells were washed with cold PBS and then stained with propidium iodide (PI, Beyotime, Shanghai, China) for 30 min at 37 °C in the dark. The stained cells were assessed using a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA).

RNA-Seq. Total RNAs were extracted using TRIzol reagent (Invitrogen). The integrity of the RNA was monitored using Bioanalyzer 2100. RNA-Seq was performed using BGISEQ-500 sequencer. A two-fold cutoff was used to identify differentially expressed genes between two cell populations. Differentially expressed genes between the two groups were identified by the NOISeq method. GO terms enrichment was analyzed using DAVID (https://david.ncifcrf.gov/) and Gene Set Enrichment Analyses (GSEA) [19, 20].

Xenograft tumor formation assay. An animal experiment protocol was approved by the ethics review committee of the Central South University of China. For nude mouse tumor cell xenograft model, tumor cells were harvested using trypsin-EDTA, washed with DMEM, resuspended in serum-free DMEM, and the suspended cells (1×10^6/0.2 ml) were subcutaneously injected into 4 to 5-week old male BALB/c nude mice (n=4; Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China). Tumor growth was monitored by measuring the tumor sizes with a caliper. The tumor volume (V) was calculated according to the formula: $V = 0.5 \times (\text{length} \times \text{width}^2)$. Nude mice were sacrificed at 12 weeks after tumor cell inoculation or when the tumor became moribund. The xenograft tumors were fixed in 4% saline-buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with H&E. A minimum of 15 sections was examined per mouse under a light microscope.

Statistical analysis. Differences between groups were estimated by using Pearson's c2 to analyze the association of NOR1 expression with clinicopathological characteristics by using the SPSS 18.0 software package (SPSS, Chicago, IL). The Kaplan-Meier method was used to determine the probability of survival and data was analyzed with the logrank test by using GraphPad Prism software (version 6, La Jolla, CA). A p-value <0.05 was considered significant.

Results

A decrease of NOR1 protein in glioma samples predicts unfavorable clinical outcome. In this study, NOR1 protein level was detected in all glioma samples and surrounding normal brain tissue samples adjacent to glioma. Strong cytoplasmic staining for NOR1 protein was detected in 45 out of 48 (93.8%) normal brain tissue samples (Figure 1A). A substantial portion of glioma samples (194 of 236; 82.2%, p<0.001) showed weak NOR1 staining (Figure 1B), but only a minority (42 of 236; 17.8%) of glioma samples displayed strong NOR1 staining (Figure 1C). This data indicated that NOR1 protein was downregulated in glioma compared to normal brain tissue. We then analyzed the relationship between NOR1 expression and clinicopathological data of glioma patients. NOR1 expression is associated with an early clinical stage in glioma patients (Table S1). Kaplan-Meier analysis revealed that low NOR1 expression was associated with poorer overall survival compared to patients with high NOR1 level in 118 glioma patients (p=0.0413 respectively, Figure 1D). There was a 40% reduction in overall survival of patients with a low level of NOR1 expression compared to those patients with a high level of NOR1 expression. Thus, our data suggested that the downregulation of NOR1 contributed to the development and progression of glioma.

Stable expression of NOR1 inhibits U251 cells proliferation *in vitro*. To study the effect of NOR1 on glioma cells, the human NOR1 gene was stably introduced into U251 cells with a NOR1 expressing lentivirus. The NOR1 expression level was determined by qRT-PCR and western blot (Figures 2A, 2B). Growth curve assays indicated that exogenous NOR1 dramatically inhibited U251 cells growth (Figure 2C). Colony formation assay revealed that the re-expression of NOR1 significantly inhibited tumor cell colony formation (p<0.001, Figures 2D, 2E). We also analyzed the effect of NOR1 expression on cell cycle progression. Flow cytometry assay revealed that overexpression of NOR1 induced cell cycle S to G2 phase arrest in U251 cells (Figure 2F). Thus, our data suggested that NOR1 acted as a tumor suppressor in glioma.

Re-expression of NOR1 suppressed migration and invasiveness progression of U251 cells. To get further insights into the biological relevance of NOR1 expression, we analyzed its effect on the migrative and invasive behavior of U251 cells. The wound-healing assay revealed that NOR1-expressing U251 cells exhibited significantly reduced mobility compared to vector control cells (Supplementary

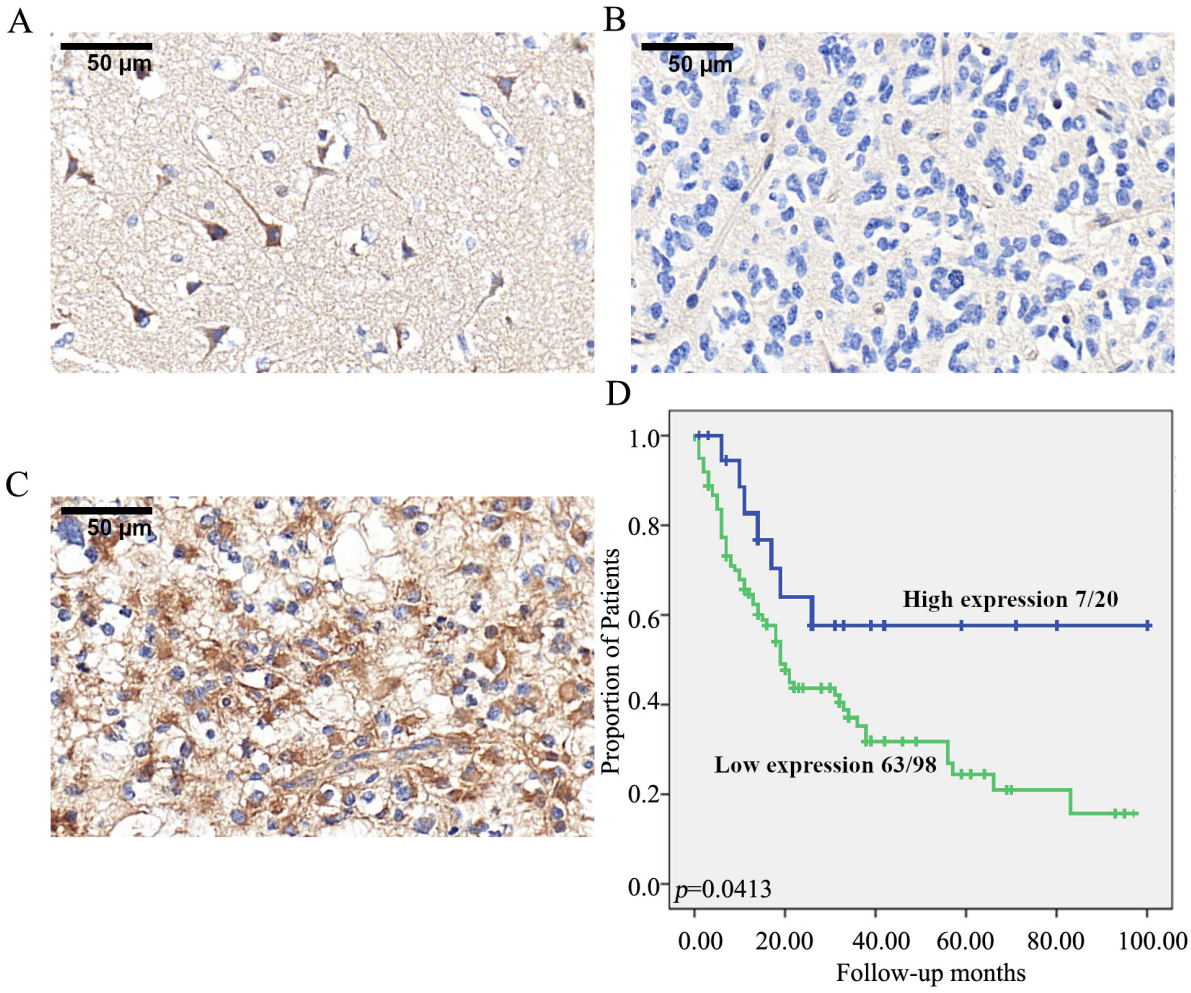

Figure 1. The loss of NOR1 protein in glioma predicts an unfavorable prognosis. A) Strong immunostaining of NOR1 protein in the normal brain. B) Weak staining of NOR1 in glioma. C) Strong staining of NOR1 in glioma. D) Overall survival of NPC patients stratified according to NOR1 protein expression. A staining color obtained as the expression intensity for NOR1 protein (color: negative = colorless, weak = yellow, moderate = brown, or strong = tawny).

Figure S1). Migration assays clearly indicated that NOR1 expression suppressed U251 cell migration (Figure 3A). We also performed the Matrigel-coated Boyden chamber invasion assay. The results demonstrated that NOR1 expression decreased glioma cell invasiveness (Figure 3B). qPCR assay showed that NOR1 expression suppressed the expression of mesenchymal markers (Vimentin, Twist, Fibronectin, Figure 3C). Thus, our data suggested that NOR1 suppressed migration and invasiveness in glioma.

Stable expression of NOR1 in U251 cells suppresses their tumorigenicity *in vivo*. We then asked whether NOR1 suppressed glioma malignant behavior *in vivo*. Human tumor xenograft models revealed that NOR1 expression suppressed the tumorigenicity of U251 cells in nude mice (Figures 4A, 4B). Xenografts from NOR1-expressing cells growled slowly and were much smaller than that from the control cells (Figures 4C, 4D). Hematoxylin and eosin staining suggested that NOR1 promoted glioma cell differentiation with a decreased nucleus-to-cytoplasm ratio, well-organized tumor architecture in glioma cells (Figure 4E). Immunohistochemical staining demonstrated fewer Ki67 cells in tumors from NOR1/U251 cells (Figures 4F, 4G). These data indicated that NOR1 suppressed tumorigenicity U251 cell *in vivo*.

NOR1 overexpression inhibits FOXR2 expression. To address the downstream effects of NOR1 expression, we performed RNA-Seq analysis on NOR1/U251 and NC/U251 cells. Using three biological replicates, we found 77 differentially regulated genes (including 34 upregulated and 43 downregulated genes) with a false discovery rate of $p<0.05$ upon NOR1 expression in U251 cells (Figure 5A). KEGG analysis revealed these differentially expressed genes correlated with metabolism (Figure 5B). GSEA analysis showed the gene expression pattern in NOR1-expressing U251 cells was correlated with the epithelial to mesenchymal transition

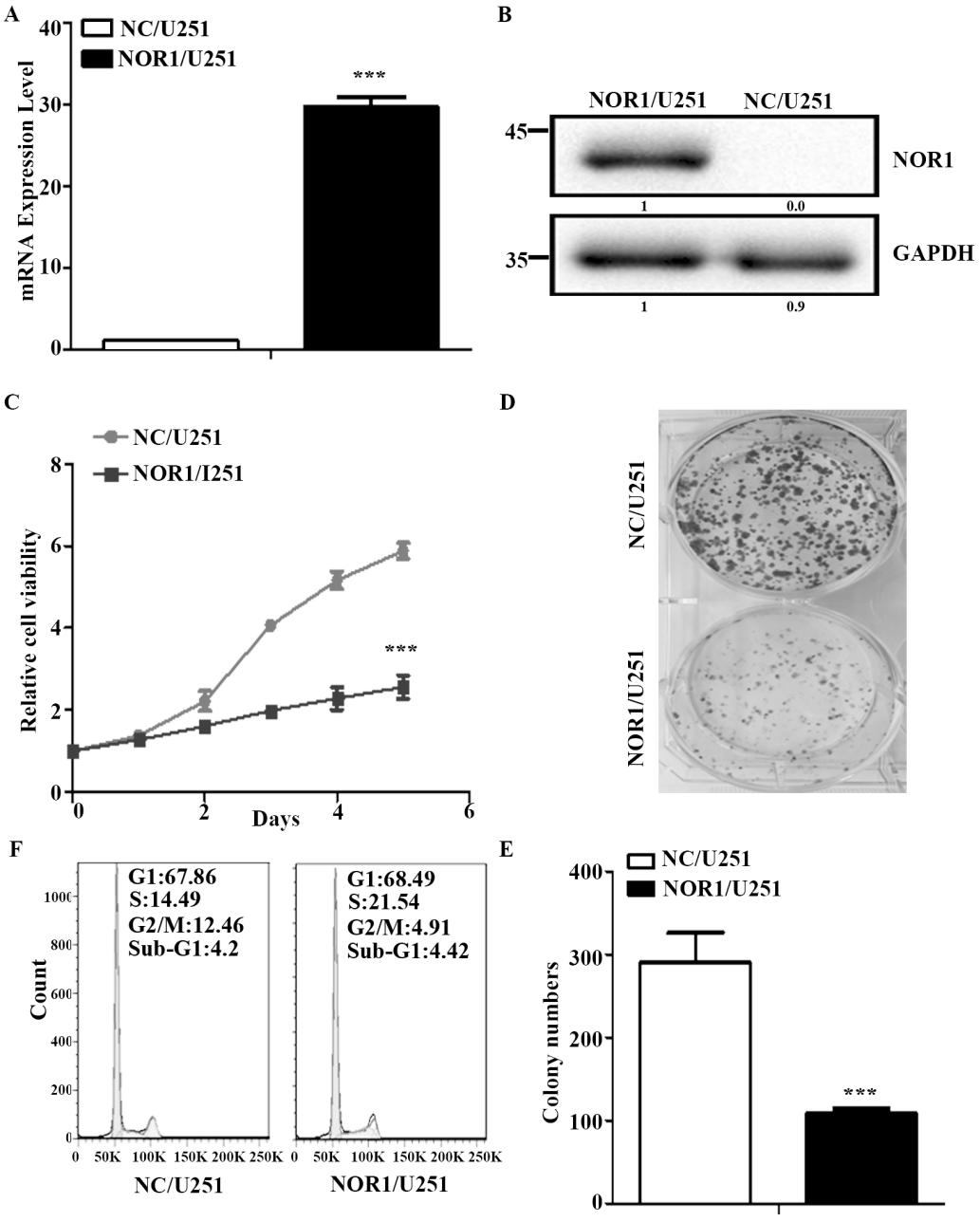

Figure 2. The re-expression of NOR1 protein suppresses glioma cell proliferation and cell cycle *in vitro*. A) NOR1 mRNA levels in U251 cells stably transfected with a NOR1-lentivirus were determined by qPCR. B, the NOR1 protein levels in U251 cells were measured by western blotting. C) CCK8 assay showed that NOR1 overexpressing inhibited cell growth. D, E) Colony-forming ability of U251 cells with stable-express of NOR1. F) Cell cycle analysis showed S/G2 arrest in U251 cells after re-expressing of NOR1. ***p<0.001 compared to the control cell.

progression signaling pathway (Figure 5C). We evaluated the RNA-Seq data by RT-qPCR (Figure 5D). As shown in Figure 5D, FOXR2, LASP1, PDGFA, and RUNX3 were dramatically reduced in NOR1-expressing U251 cells. We further demonstrated FOXR2 protein was dramatically reduced in NOR1-expressing U251 cells (Figure 5E). Immunohistochemical staining demonstrated increased FOXR2 protein in tumors from NOR1/U251 cells (Figure 5F). This result suggested that NOR1 suppressed glioma progression via modulating FOXR2 expression.

Discussion

In this study, we demonstrated that the tumor suppressor NOR1 is frequently decreased in glioma. Loss of NOR1 predicted unfavorable clinical outcomes. NOR1 suppressed

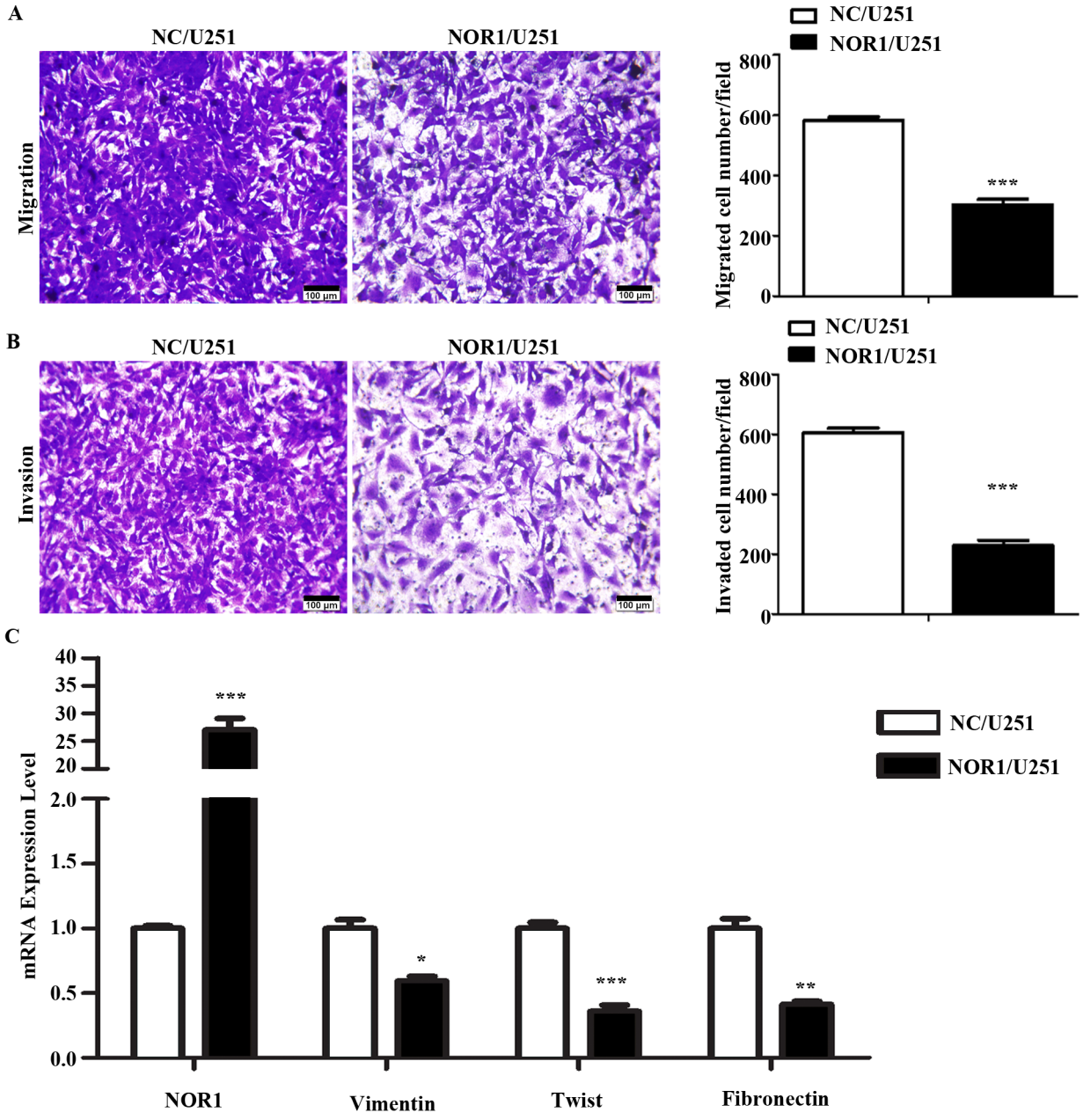

Figure 3. The re-expression of NOR1 protein suppresses glioma cell migration and invasiveness. A) Migration assay showed re-expressing NOR1 inhibits U251 cell migration. B) Invasion assay showed re-expressing NOR1 inhibits U251 cell invasiveness. C) Real-time RT-PCR assay showed NOR1 differentially regulates mesenchymal relative gene expression in U251 cells. *p<0.05, **p<0.01, ***p<0.001 compared to the control cell.

glioma cell proliferation and invasiveness *in vitro*. NOR1 suppressed tumorigenicity *in vivo*. We further depicted that FOXR2, an oncogene, was downregulated by NOR1 in glioma.

NOR1, a novel gene containing a nitroreductase domain, was the first nasopharyngeal epithelial-specific tumor suppressor gene [21]. Early studies found that the coding region single nucleotide polymorphism in the coding region of the NOR1 gene is associated with the pathogenesis of NPC, suggesting that the NOR1 gene is the NPC susceptibility gene [22]. Restoring NOR1 expression in HNE1 cells has the effect of inhibiting tumor cell growth and proliferation. Hypermethylation of the NOR1 gene promoter is one of the reasons for its downregulation in NPC tissues and cells. Oxidative stress can initiate NOR1 transcription by activating the transcription factors HSF1 (heat shock factor 1) and NRF1 (nuclear respiratory factor 1), inhibiting autophagy to promote apoptosis and preventing tumorigenesis [9].

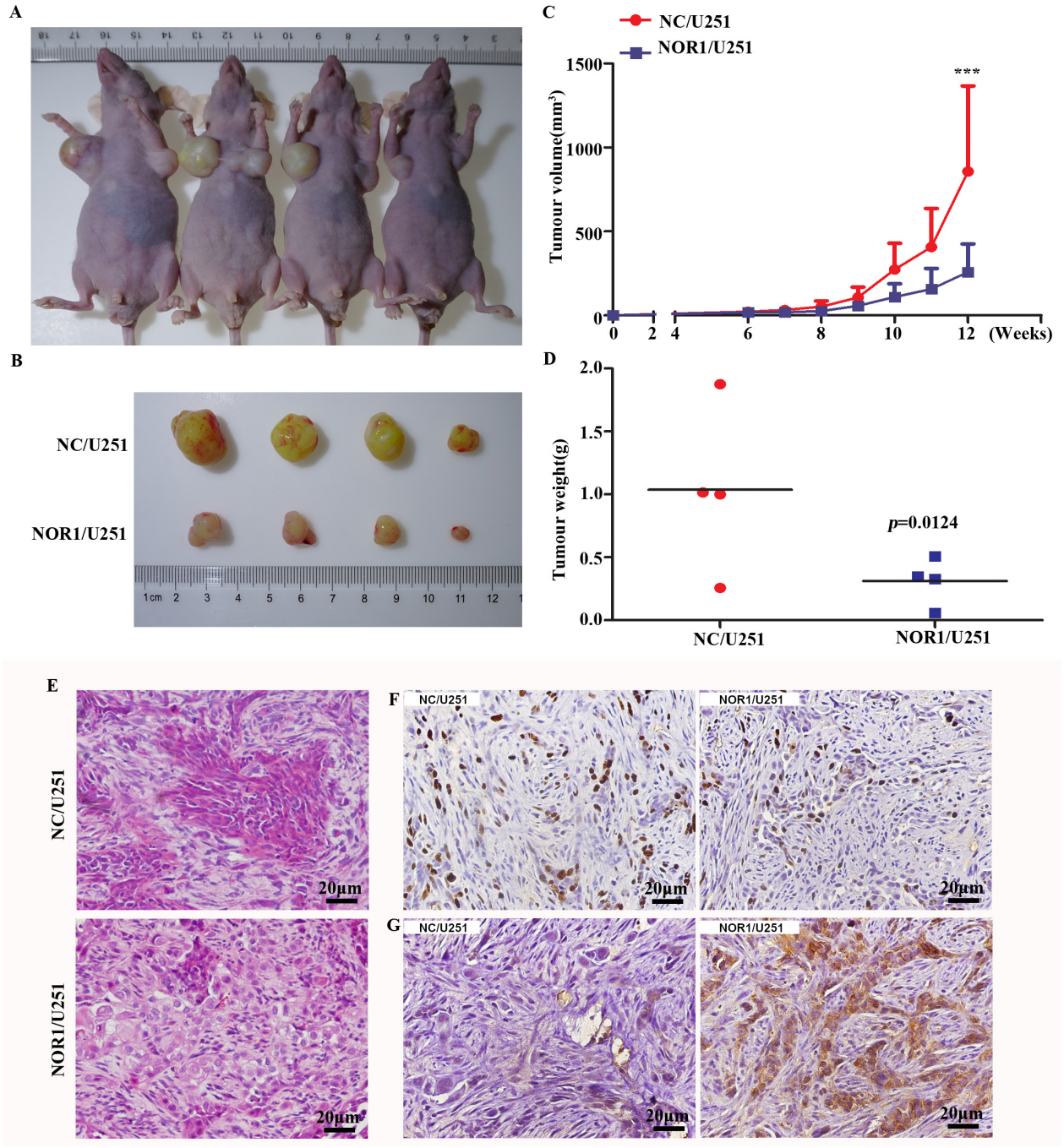

Figure 4. A stable re-expression of NOR1 suppresses tumorigenicity of glioma cells in nude mice. A) Inhibition of growth of tumor xenografts in nude mice by stable expressing of NOR1 in U251 cells. B) Comparison of tumor weight between the control and NOR1-expressing groups. C) Growth curve showing the effect of NOR1-expressing on xenograft growth in nude mice. D) Tumor weight showing the effect of NOR1-expressing on xenograft growth in nude mice. E, H) Staining of xenograft tissues from the control and NOR1-expressing groups. F, G) Immunohistochemical staining showed Ki67 and NOR1 expression *in vivo*. A staining color obtained as the expression intensity for Ki67 and NOR1 protein (color: negative = colorless, weak = yellow, moderate = brown, or strong = tawny). ***p<0.001 compared to the control cell.

NOR1 inhibits tumor cell Slug transcription by FOXA1 and HDAC2, up-regulates the expression of epithelial keratin cytokeratin 4 and cytokeratin 13, promotes cytoskeletal remodeling and inhibits epithelial-to-mesenchymal transition [23]. The above studies indicate that the NOR1 gene is a candidate for a new tumor suppressor gene.

NOR1 is conserved in the evolution of multiple species, studies have confirmed that NOR1 is highly expressed in

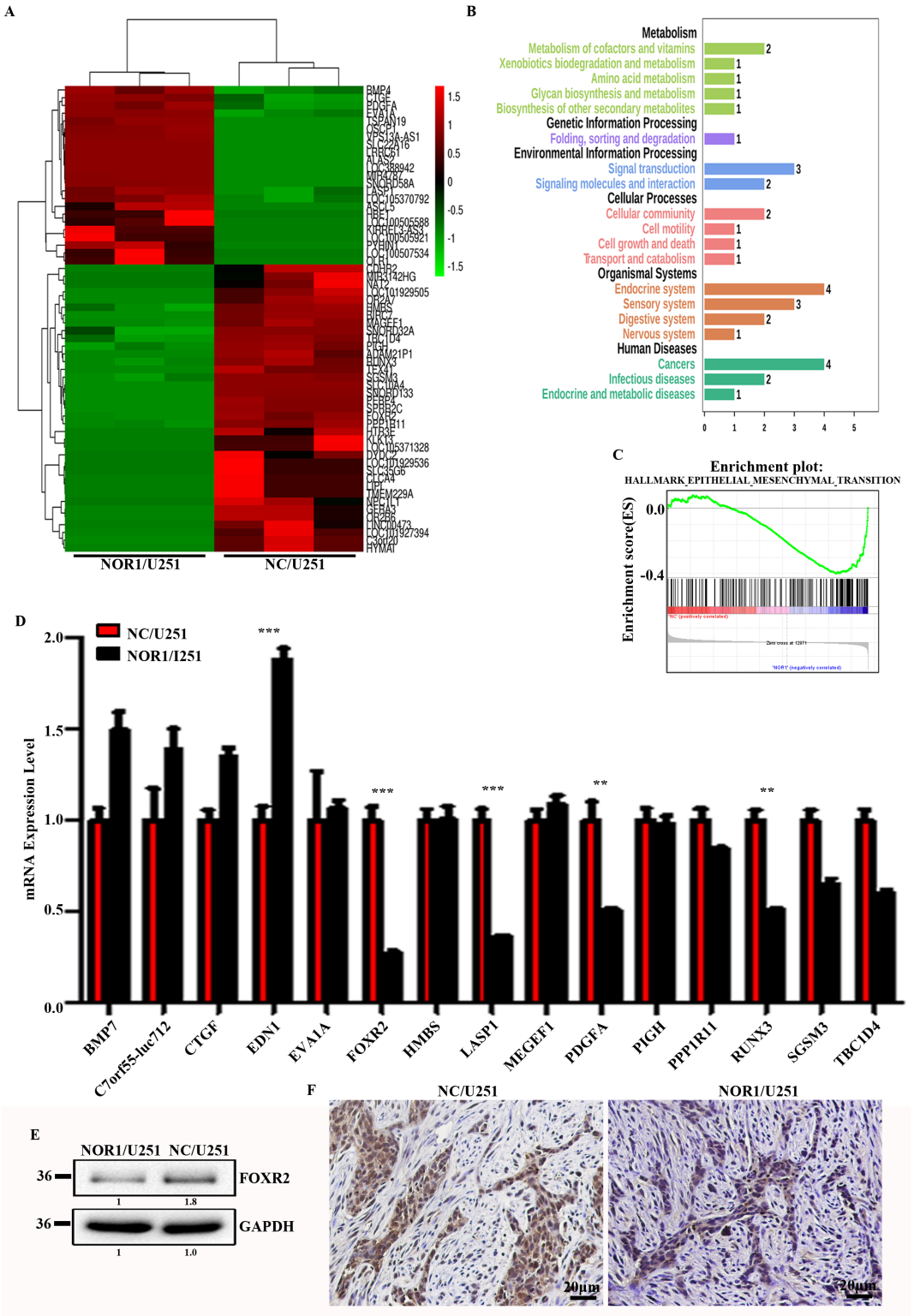

Figure 5. The re-expression of NOR1 protein suppresses glioma FOXR2 expression. A) Heatmap showed expression data for representative DEGs between NOR1-expressing or NC-U251 cells. B) KEGG analysis different expression genes. C) GSEA analysis showed RNA-Seq data from NOR1/U251 and NC/U251 cells. D) Real-time RT-PCR assay showed NOR1 differentially regulates FOXR2 expression in U251 cells. E) Western blot showed NOR1 differentially regulates FOXR2 expression in U251 cells. F) Immunohistochemical staining showed that FOXR2 expression *in vivo*. A staining color obtained as the expression intensity for FOXR2 protein (color: negative = colorless, weak = yellow, moderate = brown, or strong = tawny). *p<0.05, **p<0.01, ***p<0.001 compared to the control cell.

human brain, testis, nasopharynx, bronchial mucosa, and is hardly expressed in tissues such as heart, liver, spleen, kidney, stomach, and skeletal muscle [16]. NOR1 is highly expressed in normal brain, but low in glioma, suggesting that NOR1 plays an important role in the development of glioma.

NOR1 protein was expressed in all the brain regions at different levels, especially higher levels in neurons, suggesting a role of NOR1 in brain development. We also depicted that NOR1 was decreased in most glioma samples. Furthermore, loss of NOR1 is associated with metastasis and predicted the poor prognosis of glioma patients. Our data are similar to the observation that the loss of NOR1 expression independently predicted an unfavorable overall survival in NPC.

Human Forehead-box (FOX) gene family consists of at least 43 members [24]. Forkhead-box (FOX) superfamily genes are implicated in carcinogenesis through gene amplification, retroviral integration and chromosomal translocation [25, 26]. Forkhead box R2 (FOXR2, also named as FOXN6), a member of the FOX gene family, is associated with a variety of biological processes such as embryogenesis, differentiation, proliferation, transformation, apoptosis, tumorigenesis and so on [27]. As the transcription factor human FOXR2, FOXN6 has been identified and characterized by human genome sequence RP11-167p23 (AL159987) by bioinformatics methods [28]. By combining the results of a large-scale proteomic analysis of the human transcription factor interaction network with knowledge databases, studies identified FOXR2 as one of the top-ranked candidate proto-oncogenes. FOXR2 is highly expressed in breast cancer samples and is associated with poor prognosis. The role of FOXR2 as an oncogene in medulloblastoma, colorectal cancer [29], endometrial adenocarcinoma [30], prostate cancer [31], breast cancer [28], hepatocellular carcinoma cell [32]. In addition, FOXR2 was identified as an oncogene in malignant peripheral nerve sheath tumors and medulloblastoma and promoted the proliferation of granule neuron precursor cells [33].

In conclusion, we depicted that NOR1 is highly expressed in the brain. Loss of NOR1 protein is common in glioma. NOR1 acts as a tumor suppressor in glioma. NOR1 inhibits the proliferation and clonality of glioma cells by interacting with FOXR2, causing S to G2 arrest, inhibiting tumor migration and invasion, and reducing cell tumorigenicity. Our study provides new insight for NOR1 and a new target for the comprehensive treatment of glioma.

Supplementary information is available in the online version of the paper.

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