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The Histone Demethylase NO66 Induces Glioma Cell Proliferation

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Abstract. *Background/Aim: The histone demethylase NO66 regulates gene and protein expression. Epidermal growth factor receptor (EGFR) is a key oncogenic factor for glioblastoma. This study aimed to examine the role of NO66 in glioblastoma. Materials and Methods: The prognostic value of NO66 expression in 263 human glioma tissues and 510 glioblastoma tissues was examined by Kaplan and Meier survival analysis. Immunoblot analysis of EGFR expression, cell proliferation assays and cell cycle analysis were performed in glioblastoma cells after NO66 knockdown. Results: In 263 human glioma tissues, high levels of NO66 expression correlated with advanced disease stage and poor patient prognosis. In 510 glioblastoma tissues, high levels of NO66 expression also predicted poor patient prognosis. NO66 knockdown reduced EGFR expression and cell proliferation in glioblastoma cells. Conclusion: High levels of NO66 in glioma and glioblastoma tissues predict poor patient prognosis, and NO66 is required for EGFR expression and glioblastoma cell proliferation.*

Glioma is the most common neoplasm of the brain in adults and is one of the deadliest human cancers (1). Despite surgery, chemotherapy and radiation therapy, the median survival time for patients with stage IV glioma, also known as glioblastoma, is approximately 15 to 16 months, and most patients die from this disease within 2 years (1). There is an urgent need for better understanding of glioma tumorigenesis and the identification of novel therapeutic targets.

Histone modification enzymes, such as histone deacetylases, methyltransferases and demethylases, play important roles in the transcription of important oncogenes and the initiation and progression of various cancers (2-5). Histone deacetylase inhibitors induce the acetylation of histone and non-histone proteins, activate the transcription of tumor suppressor genes, result in glioma cell growth inhibition and apoptosis, and are currently in clinical trials in glioma patients [reviewed in (4)]. Using RNA interference (RNAi) screening technology and orthotopic patient-derived glioblastoma xenograft models, Miller *et al*. have identified the histone demethylase JMJD6 as a critical regulator of oncogene transcription and glioblastoma cell survival, and confirmed the anticancer efficacy of therapies targeting JMJD6 (6). Using CRISPR-Cas9 approaches in patient-derived glioblastoma stem cells to interrogate function of the coding genome, MacLeod *et al*. have identified the histone methyltransferase DOT1L as an actionable factor important for glioblastoma stem cell growth and temozolomide resistance (7).

The Jumonji C (JmjC)-domain containing histone lysine demethylase NO66, also known as c14orf129, is located at both nucleoli and chromatin, and is highly conserved during evolution (8). As a histone demethylase for monomethylated histone lysine 4 (H3K4me) and lysine 36 (H3K36me), NO66 plays a critical role in blocking osteoblast differentiation and skeletal formation through direct interaction with the osteoblast-specific transcription factor Osterix and inhibition of Osterix-mediated transcriptional activation (9). NO66 also binds the Polycomb repressive complex 2 (PRC2) component PHF19 at stem cell genes during stem cell differentiation, leading to PRC2-mediated trimethylation of histone H3 Lys27 (H3K27), loss of H3K36me3 and transcriptional silencing (10). In addition, NO66 catalyzes histidine hydroxylation of proteins such as 60S ribosomal protein RPL8 (11, 12), and thereby modulates protein synthesis.

The role of NO66 in glioma has not been reported. In this study, we have found that high levels of NO66 gene expression in human glioma tissues positively correlate with advanced disease stage and poor survival in a cohort of glioma patients, and high levels of NO66 gene expression in

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human glioblastoma tissues also significantly corelate with poor survival in another independent cohort of glioblastoma patients. In glioblastoma cells, NO66 knockdown reduces the expression of the key glioblastoma oncoprotein epidermal growth factor receptor (EGFR) and results in cell growth inhibition.

Materials and Methods

Cell culture and transfection. U87 and SF126 human glioblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37˚C with 5% carbon dioxide. U87 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and SF126 cells were purchased from the European Collection of Cell Cultures, respectively.

siRNAs transfection. Cells were transfected with 20 nM control siRNA or siRNAs targeting NO66 with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as described previously. Validated negative control siRNA and siRNAs specifically targeting NO66 were purchased from QIAGEN (Qiagen, Hamburg, Germany). The negative control siRNA did not target any human gene (All Stars Negative Control siRNA, Qiagen). The target sequences of NO66 siRNAs were: 5'-AACCATCTCATCTAGGTACAA-3' for NO66 siRNA-1; and 5'-AAGCAGCTGCGAAGTGTTGTA-3' for NO66 siRNA-2.

RNA extraction and RT-PCR. RNA was extracted from glioblastoma cells, 48 h after transfection with control siRNA or NO66 siRNAs, with PureLink RNA Mini Kit (Invitrogen). cDNA was synthesized from the RNA samples with M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and ~10 ng of the resulting cDNA was used in real-time PCR reactions with SYBR Green PCR Master Mix (Thermo Fisher Scientific) and gene-specific forward or reverse primer (Sigma, Sydney, Australia). The real-time PCR reactions consisted of one cycle at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 1 min. The sequences of the primers for PCR amplifications were: 5'-CACCATGTACCCTGGCATT-3' (forward) and 5'-ACGGAGT ACTTGCGCTCAG-3' (reverse) for β-actin; and 5'-GCGAGCCA AAGACTTCATTC-3' (forward) and 5'-GAATTGGAAGCCCGT AAACA-3' (reverse) for NO66.

Protein extraction and immunoblot. Forty-eight h after siRNA transfection, glioblastoma cells were washed with cold phosphate buffered saline and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5) with 1% Protease Inhibitor Cocktail (Sigma) for 30 min on ice. After centrifugation for 20 min at 13,000 × *g* at 4˚C, the supernatants were recovered and protein concentration was determined by the Bicinchoninic Acid protein assay kit (Thermo Fisher Scientific). Thirty μg of total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to membrane, followed by blocking for one h with 10% skim milk. Membranes were probed with rabbit anti-NO66 antibody (1:2000, Abcam, Cambridge, MA, USA), mouse anti-EGFR antibody (1:500, Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-c-Myc antibody (1:1000, Abcam), and mouse anti-actin antibody (1:10000, Sigma), followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:10000, Merck, Kenilworth, NJ, USA). Protein expression was imaged using SuperSignal reagents (Thermo Scientific).

Alamar blue assays. Cell proliferation was examined with Alamar blue assays. Briefly, glioblastoma cells were plated into 96-well plates, transfected with control siRNA, NO66 siRNA-1 or NO66 siRNA-2. Ninety-six h after siRNA transfection, cells were incubated with 20 μl per well of Alamar blue reagent (Invitrogen) for 6 h, and then plates were read on a microplate reader at 570/595 nm. Results were analysed by calculating the optical density absorbance and expressed as percentage change in cell numbers, as we described previously (13, 14).

Cell cycle analysis. U87 and SF126 glioblastoma cells were transfected with control siRNA, NO66 siRNA-1 or NO66 siRNA-2. Ninety-six hours later, the cells were harvested, pelleted, washed and fixed drop-wise in ice-cold 70% ethanol. Cells were then stained with propidium iodide (Sigma) and DNAse-free RNAse (Sigma), and propidium iodide staining in the cells was examined by FACSCallibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells at each phase of the cell cycle was analysed using FlowJo software (Tree Star Incorporated, Ashland, OR, USA).

Patient tumor sample analysis. NO66 mRNA expression was examined using the publicly available Glioma - French dataset (Tumor Glioma - French - 284 - MAS5.0 - u133p2) (15, 16) and Glioblastoma - The Cancer Genome Atlas (TCGA) dataset (Tumor Glioblastoma-TCGA-540-MAS5.0-u133a) (17), both downloaded from the R2: Genomics Analysis and Visualization Platform (18). Only 263 out of 284 samples in the Glioma - French dataset and 510 out of 540 samples in the Glioblastoma - TCGA dataset were used for analysis, as information on patient prognosis for the other samples was not available. Correlation between NO66 gene expression and disease stage (stages I, II and III *versus* stage IV) in the 263 glioma tumor tissues was analysed with un-paired Student's *t*-test. Probability of survival was investigated according to NO66 gene expression levels in the 263 glioma and 510 glioblastoma tissues, using the Kaplan–Meier method and the two-sided log-rank test (19).

Statistical analysis. Results are expressed as the mean and standard errors. The paired Student's *t*-test and one-way ANOVA were used to determine statistical significance between two groups or more than two groups respectively. A p-value of <0.05 was considered statistically significant.

Results

High levels of NO66 expression in glioma tissues correlate with advanced disease stage and poor patient prognosis. To assess the clinical relevance of the histone demethylase NO66 in glioma, we examined NO66 gene expression in human glioma and glioblastoma tissues in the publicly available Glioma - French dataset (Tumor Glioma - French - 284 - MAS5.0 - u133p2) (15, 16) and Glioblastoma - TCGA dataset (17), both downloaded from the R2: Genomics Analysis and Visualization Platform (18). Un-paired Student's *t*-test showed

that NO66 gene expression was significantly higher in 150 stage IV, compared with 113 Stage I, II and III, glioma tissues in the Glioma - French dataset (Figure 1A). Importantly, Kaplan–Meier survival analysis showed that high NO66 gene expression levels were associated with poor patient prognosis in the 263 glioma tissues of the Glioma - French dataset and the 510 glioblastoma tissues of the Glioblastoma - TCGA dataset (Figure 1B). Taken together, the data suggest that high levels of NO66 expression in human tumor tissues correlate with advanced glioma stage and predict poor prognosis in glioma and glioblastoma patients.

NO66 mRNA and protein are effectively knocked down by two independent siRNAs. We next transfected U87 and SF126 glioblastoma cells with control siRNA, NO66 siRNA-1 or NO66 siRNA-2. The two NO66 siRNAs targeted different regions of the NO66 mRNA. As shown in Figure 2A, RT-PCR analysis confirmed that transfection with the two independent NO66 siRNAs efficiently knocked down NO66 mRNA expression. Consistent with these data, immunoblot analysis demonstrated that transfection with NO66 siRNA-1 or NO66 siRNA-2 efficiently knocked down NO66 protein expression (Figure 2B). The data therefore confirm the efficacy of the two independent NO66 siRNAs.

NO66 is required for EGFR and c-Myc expression in glioblastoma cells. EGFR is a key glioblastoma oncoprotein (20) and c-Myc also plays a critical role in glioblastoma tumorigenesis (21). We examined whether NO66 modulated EGFR and c-Myc protein expression. As shown in Figure 3, immunoblot analysis demonstrated that knocking down NO66 with the two independent siRNAs both effectively reduced EGFR and c-Myc protein expression in U87 and SF126 cells. The data confirm that NO66 is required for EGFR and c-Myc oncoprotein expression in glioblastoma cells.

NO66 promotes glioblastoma cell proliferation. To understand whether NO66 plays a role in glioblastoma cell proliferation, we transfected U87 and SF126 glioblastoma cells with control siRNA, NO66 siRNA-1 or NO66 siRNA-2. Alamar blue assays showed that transfection with the two independent NO66 siRNAs, compared with the control siRNA, significantly reduced the number of glioblastoma cells (Figure 4A).

We next transfected U87 and SF126 cells with control siRNA, NO66 siRNA-1 or NO66 siRNA-2 for 72 h, followed by staining with propidium iodide. Flow cytometry analysis showed that knocking down NO66 with NO66 siRNAs significantly decreased the percentage of cells at the S phase (Figure 4B). The data demonstrate that NO66 expression in glioblastoma cells induces glioblastoma cell proliferation.

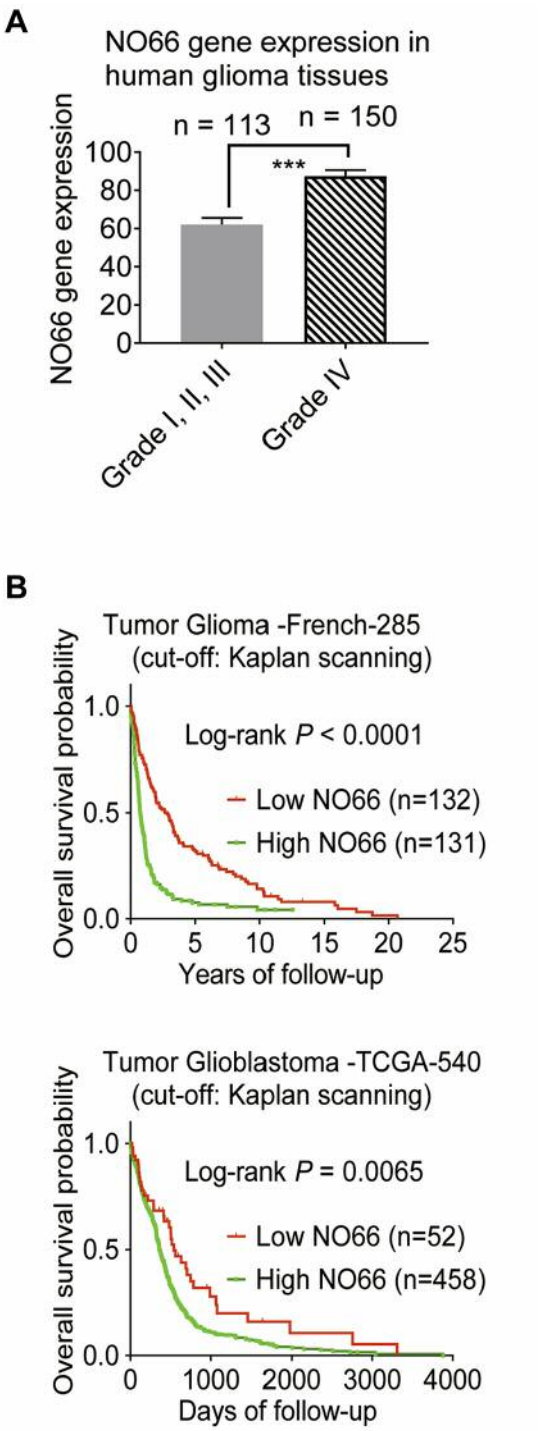

Figure 1. *High levels of NO66 expression in glioma tissues correlate with advanced disease stage and poor patient prognosis. Correlation between NO66 gene expression and disease stage (stages I, II and III versus stage IV) in the 263 glioma tumor tissues in the publicly available Glioma - French dataset was analysed with paired Student's t-test (A). Kaplan–Meier curves showed the overall survival probability of patients according to NO66 mRNA expression levels in the 263 glioma tissues in the publicly available Glioma - French dataset and 510 glioblastoma tissues in the publicly available Glioblastoma - TCGA dataset (B).*

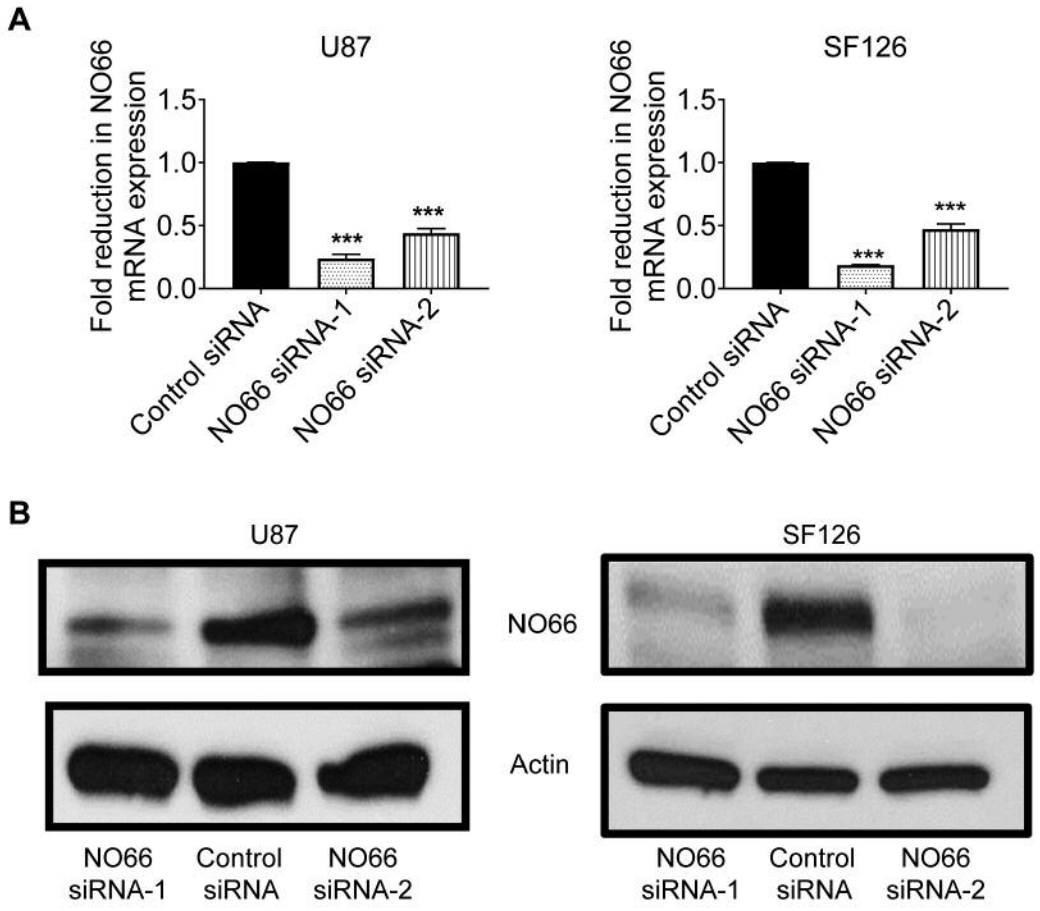

Figure 2. *NO66 mRNA and protein are effectively knocked down by two independent siRNAs. U87 and SF126 cells were transfected with control siRNA, NO66 siRNA-1 or NO66 siRNA-2 and incubated for 48 h. RNA was extracted from the cells for RT-PCR analysis of NO66 mRNA expression (A), and protein was extracted from the cells for immunoblot analysis of NO66 protein expression (B). Error bars represent standard error. *p<0.05, **p<0.01, and ***p<0.001.*

Discussion

The histone demethylase NO66 binds transcription factors, such as Osterix and PHF19, targets H3K4me, H3K27me3, H3K36me3 and H3K36me, and thereby regulates gene transcription (9, 10, 22). By catalyzing histidine hydroxylation of the 60S ribosomal protein RPL8, NO66 post-transcriptionally modulates protein synthesis (11, 12). Through regulating gene and protein expression, NO66 plays an important role in cellular functions.

NO66 is selectively expressed in human colorectal cancer tissues, and high NO66 expression in human colorectal cancer tissues is associated with poor patient prognosis and metastasis, including lymphatic duct invasion, venous invasion and lymph node metastasis (23). NO66 is also highly expressed in human prostate cancer tissues, and high levels of NO66 expression in prostate cancer tissues predict poor patient prognosis (22).

The role of NO66 in glioma has not been reported. In this study, we have found that high levels of NO66 gene expression in human glioma tissues positively correlate with advanced disease stage and poor survival in a cohort of glioma patients, and high levels of NO66 gene expression in human glioblastoma tissues also significantly corelate with poor survival in another independent cohort of glioblastoma patients. The data confirms NO66 as a marker for poor prognosis in glioma and glioblastoma patients.

NO66 has most recently been reported to activate the expression of the pro-oncogenic protein β-catenin and cyclin D1, the cell proliferation and survival protein MCL1, the invasion-associated proteins IGFBP5 and MMP3, and the epigenetic modifier KMT2A in androgen-independent prostate cancer cells (22). NO66 thereby promotes prostate cancer cell proliferation *in vitro* and prostate cancer progression in mice (22). In addition, NO66 induces colorectal cancer cell proliferation and migration, although the mechanism is not clear (23).

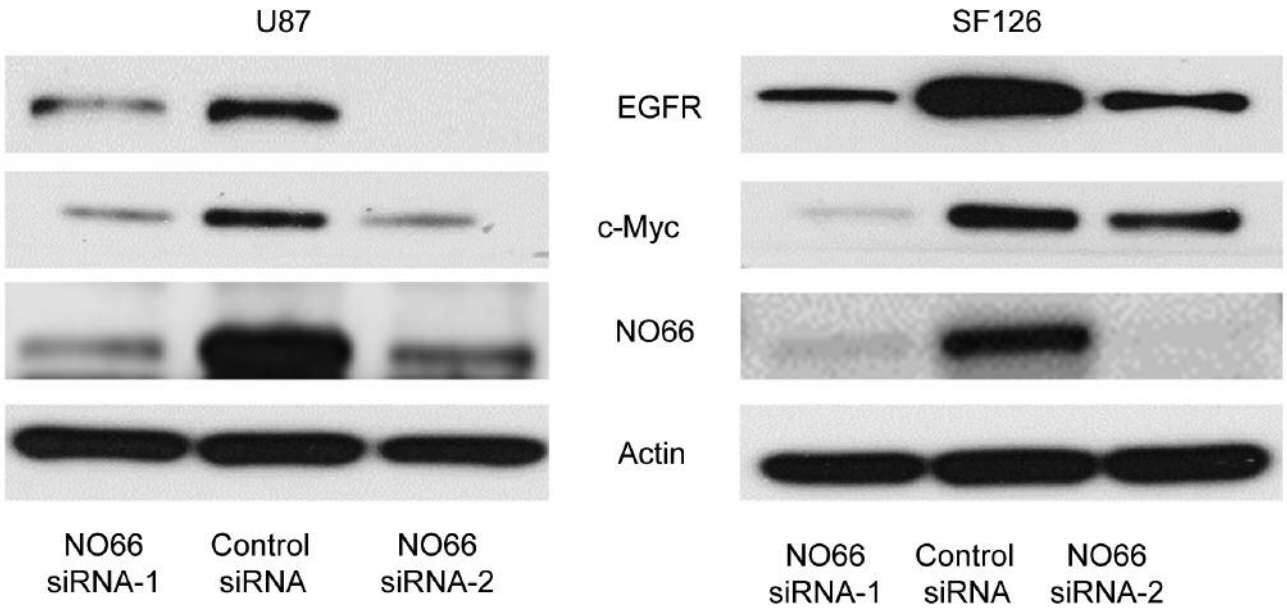

Figure 3. *NO66 is required for EGFR protein expression. U87 and SF126 cells were transfected with control siRNA, NO66 siRNA-1 or NO66 siRNA-2 and incubated for 48 h. Protein was extracted from the cells for immunoblot analysis of EGFR and c-Myc protein expression.*

EGFR activates the phosphoinositide-3-kinase/Akt (PI3K/AKT) pathway as well as the Ras/RAF/mitogen-activated protein kinase/extracellular signal-regulated (Ras/RAF/MAPK/ERK) pathway, blocks glioblastoma tumor-initiating cell differentiation, induces glioblastoma tumor-initiating cell tumorigenicity, and induces glioblastoma cell proliferation and survival (20, 24). In glioblastoma cells, silencing of the p53 and PTEN tumor suppressors leads to c-Myc oncoprotein functional activation, and c-Myc blocks glioblastoma cell differentiation, enhances self-renewal capacity of neural stem cells and glioblastoma tumor-initiating cells, and induces glioblastoma cell proliferation and tumorigenesis (21, 25). While the role of NO66 in glioblastoma has not been reported, in this study, we have found that NO66 gene knockdown with two independent siRNAs significantly reduces EGFR and c-Myc protein but not mRNA expression, and down-regulates glioblastoma cell proliferation. As NO66 is known to regulate protein synthesis through catalyzing histidine hydroxylation of the 60S ribosomal protein RPL8 (11, 12), NO66 is likely to up-regulate EGFR and c-Myc protein expression through modulating protein synthesis, and induces glioblastoma cell proliferation through regulating EGFR and c-Myc protein expression. Our data therefore identify the histone demethylase NO66 as an oncogenic factor and a therapeutic target for glioblastoma.

Conclusion

Histone modification proteins are emerging as critical regulators of tumorigenesis and as important therapeutic targets. The histone demethylase NO66 has most recently been shown to play an oncogenic role in prostate and colorectal cancers. In the currently study, we have demonstrated that high levels of NO66 expression in human glioma tissues correlate with advanced disease stage, and high levels of NO66 expression in glioma and glioblastoma tissues predict poor patient prognosis. Additionally, NO66 is required for EGFR and c-Myc oncoprotein expression, and induces glioblastoma cell proliferation. Our data therefore confirm the oncogenic role of the histone demethylase NO66 in glioblastoma, and identify NO66 as a therapeutic target for glioblastoma.

Conflicts of Interest

The Authors have no conflicts of interest to declare in regard to this study.

Authors' Contributions

QW performed the experiments, analyzed the data and wrote the first draft of the article. TL and QL conceptualized the project, supervised the experiments and revised the article.

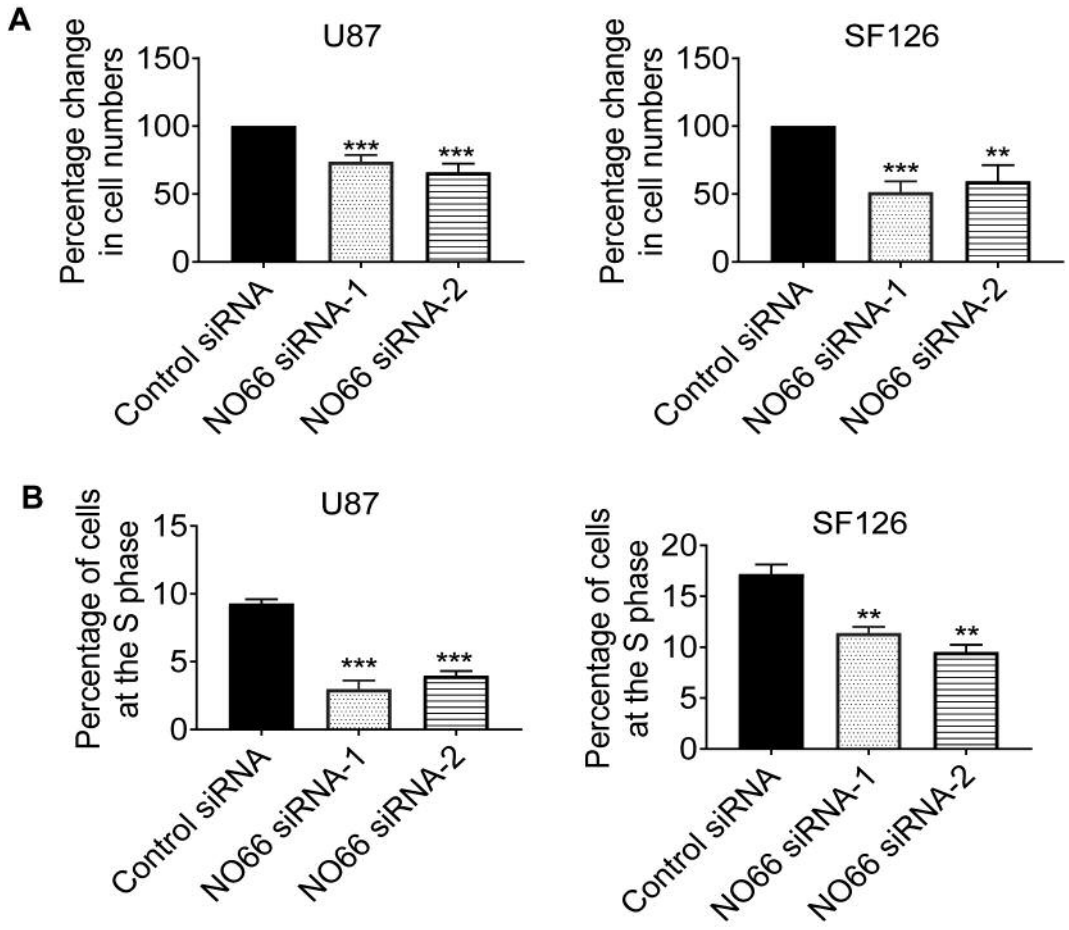

Figure 4. *NO66 promotes glioblastoma cell proliferation. U87 and SF126 glioblastoma cells were transfected with control siRNA, NO66 siRNA-1 or NO66 siRNA-2 and incubated for 96 h. Cells were then stained with Alamar blue for Alamar blue assays of the number of viable cells (A), or stained with propidium iodide for flow cytometry analysis of the cell cycle (B). For Alamar blue assays, results were expressed as percentage change of the number of viable cells, relative to control siRNA-transfected samples (A). For the flow cytometry analysis of the cell cycle, the percentage of cells at each phase of the cell cycle was calculated (B). Error bars represent standard error. ** and *** indicate p<0.01 and 0.001 respectively.*

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