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Knockdown of NOB1 expression by RNAi inhibits cellular proliferation and migration in human gliomas

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ABSTRACT

NOB1 (NIN1/RPN12 binding protein 1 homolog), a ribosome assembly factor, is thought to be essential for the processing of the 20S pre-rRNA into the mature 18S rRNA. It is also reported to participate in proteasome biogenesis. However, the contribution of NOB1 gene dysfunction to the pathology of human diseases, such as gliomas, has not been addressed. Here, we detected expression levels of NOB1 mRNA in U251, U87, U373, and A172 cells by quantitative real-time PCR. To analyze the expression levels of NOB1 protein in glioma tissues, we performed immunohistochemistry on 56 pathologically confirmed glioma samples (7 Grade I cases, 19 Grade II cases, 16 Grade III cases, and 14 Grade IV cases). A recombinant lentivirus expressing NOB1 short hairpin RNA (shNOB1) was constructed and infected into U251 and U87-MG human glioma cells. We found that NOB1 mRNA was expressed in all four cell lines. The expression level of the NOB1 protein was significantly higher in high-grade gliomas than in low-grade gliomas. Knockdown of the NOB1 gene resulted in suppression of the proliferation and the colony-forming abilities of U251 and U87-MG cells, cell cycle arrest during the G_0/G_1 phase, and a significant enhancement of cell apoptosis. In addition, cell migration was significantly suppressed in U251 and U87-MG cells that were infected with the shNOB1-expressing lentivirus. These results suggest that NOB1 promotes glioma cell growth and migration and could be a candidate for molecular targeting during gene therapy treatments of glioma.

1. Introduction

Gliomas are the most common neurological nerve cancers worldwide, accounting for 44.69% of intracranial tumors. Glioblastomas, in particular, are high-grade gliomas, accounting for 70% of gliomas (Cancer Genome Atlas Research Network, 2008; Wen and Kesari, 2008). Numerous studies have shown that gliomas develop as a result of genetic alterations that accumulate with tumor progression and therefore show a great morphological and genetic heterogeneity. Additionally, primary and secondary glioblastomas are distinct entities that evolve through different genetic pathways and that have different molecular profiles and responses to therapy (Pollo, 2011). Therefore, it is necessary to identify new therapeutic molecular targets and therapeutic strategies for the prevention and treatment of gliomas.

The human NOB1 gene is located on the human chromosome 16q22.1 and contains a putative open reading frame of 1239 bp. NOB1 mRNA is expressed mainly in the liver, lung and spleen, and the NOB1 protein is mainly localized to the nucleus of mammalian cells (Zhang et al., 2005). As a ribosome assembly factor, NOB1 is argued to be essential for the processing of the 20S pre-rRNA into the mature 18S rRNA (Fatica et al., 2003; Fatica et al., 2004; Lamanna and Karbstein, 2011; Pertschy et al., 2009). In 2009, Lamanna and Karbstein (2009) showed that the Nob1 PIN domain binds to the single-stranded cleavage site D at the 3′-end of the 18S rRNA. Recent studies also reached the same conclusion through their study of the archaeal endonuclease Nob1 in 2011 (Veith et al., 2012). These data strengthen the proposal that Nob1 is the endonuclease required for D site cleavage. At the same time, recent biochemical and genetic studies have revealed that Nob1p plays a role in the formation of the 26S proteasome and the maturation of the 20S proteasome in eukaryotes. Additionally, Tone et al. (2000) found that Nob1p, an essential and short-lived protein, interacts with Nin1p/Rpn12, a subunit of the 19S regulatory particle of the 26S proteasome, in growing *Saccharomyces cerevisiae* cells during a two-hybrid screen. Subsequent studies showed that Nob1p serves as a chaperone to help join the 20S proteasome with the 19S regulatory particle in the nucleus and facilitates the maturation of the 20S proteasome (Tone and Toh, 2002).

Abbreviations: NOB1, NIN1/RPN12 binding protein 1 homolog; RNAi, RNA interference; PIN domain, Homologues of the pilT N-terminal domain; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde3-phosphate dehydrogenase; HEK, Human embryonic kidney; ATCC, American Type Culture Collection; WHO, World Health Organization; DAB, 3,3′-diaminobenzidine; BSA, Bovine serum albumin; TRITC, Tetraethyl rhodamine isothiocyanate; shRNA, Short-hairpin RNA; siRNA, Small interfering RNA; MOI, Multiplicity of infection; M-MLV-RT, M-MLV Reverse Transcriptase; BCA, Bicinchoninic acid; MTT, Methylthiazoletetrazolium; PI, Propidium iodide; FACS, Fluorescence-activated cell sorting; IHC, Immunohistochemistry; GFP, Green Fluorescent Protein; PSMD8BP1, 26S proteasome non-ATPase regulatory subunit 8 binding protein 1.

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A growing body of evidence suggests that the ribosome (Nazar, 2004) and the 26S proteasome (Mani and Gelmann, 2005) play significant roles in the protein synthesis and ubiquitinated protein degradation pathways, respectively, which have close relationships with the development of human diseases, especially cancer. The importance of NOB1 to the process of ribosome synthesis and the maturation and formation of the 26S proteasome suggest a role for NOB1 in the development of human malignancies. Previous studies have shown that NOB1 plays an important role in the inhibition of tumor proliferation in human ovarian cancer, hepatocellular carcinoma and leukemia (Lin et al., 2012; Lu et al., 2012; Oehler et al., 2009). However, the importance of NOB1 in human gliomas is largely unknown.

To study the potential role of NOB1 in gliomas, we began with an immunohistochemistry analysis of NOB1 protein in glioma tumors and an immunofluorescence analysis of its subcellular localization in U251 glioma cells. Then, a loss-of-function analysis was performed by applying a NOB1 short hairpin RNA (shNOB1)-expressing lentivirus (Lv-shNOB1) to two glioma cell lines, U251 and U87-MG. The effect of NOB1 knockdown on glioma cell proliferation, formation of colonies, cell-cycle progression, and cell migration ability was investigated.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Logan, Utah, USA). Lipofectamine 2000 and TRIzol® Reagent was purchased from Invitrogen (Carlsbad, CA, USA). M-MLV Reverse Transcriptase was purchased from Promega (Madison, WI, USA; cat. M1705). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used were the following: anti-NOB1 (1:500 dilution; Abcam) and anti-GAPDH (glyceraldehyde3-phosphate dehydrogenase, 1:3,000 dilution; Santa Cruz Biotechnology, Inc.).

2.2. Cell culture

U373, U251, A172 and U87-MG human glioma cell lines and the human embryonic kidney (HEK) 293 T cell line were obtained from American Type Culture Collection (ATCC). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Immunohistochemistry

Before the study began, written informed consent was obtained from all patients who participated in the study, which was approved by the Ethics Committee of the Second Affiliated Hospital of Anhui Medical University. Fifty-six glioma samples were obtained from March 2010 to September 2011 from the Department of Neurosurgery at the Second Affiliated Hospital of Anhui Medical University (7 Grade I cases, 19 Grade II cases, 16 Grade III cases, and 14 Grade IV cases; grades were determined according to the 2007 WHO classification of tumors of the central nervous system). The patients included 34 males and 22 females. The mean ages of the patients at the time of surgery were 44 (male) and 41 (female). All tumors were from patients with newly diagnosed gliomas who had received no therapy before sample collection.

Immunoperoxidase staining was performed on 2 μm paraffin sections. All sections were treated with 0.3% H_2O_2 to exhaust endogenous peroxidase activity. Avidin/biotin blocking solutions were used to prevent the non-specific binding of possible endogenous biotin- or avidin-binding proteins. Blocking with 10% normal goat serum was performed before applying the primary antibody against NOB1 (1:150, Cat #SAB2101613, Sigma-Aldrich). Biotinylated goat anti-rabbit IgG was used as the secondary antibody. The immunoreactions were detected by staining with 3,3′-diaminobenzidine (DAB). For all cases, representative pictures (20× magnification) of selected regions in the NOB1-positive sections were taken with a microscope. Negative controls were performed by omitting the primary antibody.

2.4. Immunofluorescence

U251 cells grown on cover slips were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100/PBS for 20 min. SMMC-7721 cells served as a positive control. Cells were then washed twice with PBS and blocked in 3% bovine serum albumin (BSA) for 30 min prior to incubation with primary antibodies recognizing NOB1 for 1 h in a humidified chamber. After several PBS washes, cells were incubated with tetraethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies for 1 h. After washing with PBS, cells were stained with DAP (Sigma-Aldrich) for 15 min and immunofluorescence was detected using a fluorescence microscope.

2.5. Construction of a NOB1 shRNA lentivirus vector and cell infection

The following oligonucleotides were synthesized. The negative control small interfering RNA (siRNA) was 5′-TTCTCCGAACGTGTCACGT-3′, which does not target any genes in humans, mice or rats as determined by screening with NCBI RefSeq. NOB1 siRNAs were 5′-CCAAGGAA GTGCAATTGCATA-3′ and 5′-CGGGAAGAACATTTACACCAT-3′ that targets nucleotides 85–105 and 1464–1484 of the NOB1 mRNA, respectively. The stem–loop–stem oligos (short-hairpin RNAs, shRNAs) were synthesized, annealed, and ligated into the Nhe I/Pac I-linearized pFH-L vector. The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing. The generated plasmids were named pFH-L-shNOB1 or -shCon. Recombinant lentiviral vectors and packaging vectors were then transfected into 293 T cells. Supernatants containing either the lentivirus expressing the NOB1 shRNA or the control shRNA were harvested 72 h after transfection. The lentiviruses were purified using ultracentrifugation, and the titer of the lentiviruses was determined. U251 and U87-MG cells were infected with the lentivirus constructs at a multiplicity of infection (MOI) of 10, and mock-infected cells were used as negative controls.

2.6. RT-PCR

Total RNA was extracted from U251 cells 5 days after infection with the lentivirus constructs using TRIzol® Reagent. cDNA was synthesized using M-MLV Reverse Transcriptase. In brief, a mixture containing 1.5 μg of total RNA, 0.75 μg oligo-dT primer (Shanghai Sangon) and nuclease-free water in a total volume of 13.5 μl was heated at 70 °C for 5 min and then cooled on ice for another 5 min. The mixture was supplemented with 4 μl M-MLV buffer, 1.25 μl dNTP, 0.5 μl RNasin and 0.75 μl M-MLV-RT up to a final volume of 20 μl, followed by incubation at 42 °C for 60 min.

2.7. Real-time PCR analysis

Real-time quantitative PCR analysis was performed using a SYBR Green Master Mix Kit on a BioRad connect Real-Time PCR platform. In brief, each PCR reaction mixture, containing 10 μl of 2× SYBR GreenMaster Mix, 1 μl of sense and antisense primers (5 μmol/μl) and 1 μl of cDNA (10 ng), was run for 45 cycles with denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s in a total volume of 20 μl. For relative quantification, $2^{-\Delta\Delta CT}$ was calculated and used as an indication of the relative expression levels, which was calculated by subtracting the CT values of the control gene from the CT values of NOB1. The primer sequences for the PCR amplification of the NOB1 gene were 5′-AAGTGAGGAGGAGGAGGAG-3′ and 5′-ACTTTCT TCAGGGTCTTGTTC-3′. β-actin was used as an internal control. The primer sequences for β-actin were 5′-GTGGACATCCGCAAAGAC-3′ and 5′-AAAGGGTGTAACGCAACTA-3′.