Downregulation of NOB1 inhibits proliferation and promotes apoptosis in human oral squamous cell carcinoma

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Abstract. NIN1/RPN12 binding protein 1 homolog (NOB1) facilitates the maturation of the 20S proteasome and is then degraded by 26S proteasome to complete 26S proteasome biogenesis. It also accompanies the pre-40S ribosomes during nuclear export and is cleaved at D-site of 20S pre-rRNA to form mature 18S rRNA in growing cells. NOB1 was reported to be involved in the development of several types of cancer. However, the role of NOB1 in oral squamous cell carcinoma (OSCC) has not been addressed. In the present study, the expression of NOB1 in 50 OSCC patients with different genders, ages, TNM and pathological grades was detected using immunohistochemistry and western blotting. A loss-of-function study was carried out by the lentivirus-mediated siRNA knockdown of NOB1 in the CAL27 and TCA-8113 OSCC cell lines. The results showed that, NOB1 expression increased with pathological grades. In the CAL27 and TCA-8113 cell lines, knockdown of NOB1 in OSCC cells decreased cell proliferation, colony formation, increased cell apoptosis and also induced cell cycle arrest in the S phase. The results suggested that NOB1 is important in OSCC development and serves as a candidate indicator of aggressiveness and a therapeutic target of OSCC.

Introduction

Cancers of the oral cavity are common tumors in male and female patients with an estimated incidence of 264,000 and 128,000 mortalities worldwide in 2008 (1). Oral squamous cell carcinoma (OSCC) constitutes ~90% of oral malignancies (2). Chronic alcohol abuse, tobacco use and HPV infection are the most important risk factors for the development of OSCC. Smoking and alcohol synergistically contribute to the disease (1). Therefore, OSCC occurs most frequently in middle-aged to elderly patients who smoke and drink heavily. However, the incidence rates of OSCC in young adults in US and some European countries are on the increase, which is possibly caused by HPV infection, which may partly be attributed to oral sexual behavior (3).

NIN1/RPN12 binding protein 1 homolog (NOB1) was first identified in *Saccharomyces cerevisiae* by two-hybrid screening. NOB1 encodes an essential protein Nin one binding protein (NOB1p) in growing cells (4). As a nucleoprotein, NOB1p forms a complex with the 19S regulatory particle in the nucleus by binding at NIN1/RPN12, a subunit of the 19S regulatory particle of the yeast 26S proteasome and facilitates the maturation of the 20S proteasome and is then degraded by 26S proteasome to complete 26S proteasome biogenesis (5). NOB1p was also found to accompany the pre-40S ribosomes during nuclear export and be cleaves at the D-site of 20S pre-rRNA to form mature 18S rRNA (6-8). The human *NOB1* gene is localized on chromosome 16q22.1 and expresses an ~50 kDa protein NOB1. NOB1 is expressed mainly in the liver, lung and spleen in human and is mainly localized in the nucleus (9,10).

The function in ribosome assembly and proteasome biogenesis of NOB1 suggest that NOB1 may be associated with protein homeostasis and may play important roles in mediating certain physiological and pathological functions. Specifically, ribosome assembly and ubiquitin-proteasome pathway were involved in certain types of cancer (11-13). Dysfunction of the *NOB1* gene was reported to contribute to certain human cancers. Upregulation of NOB1 was first identified in esophageal squamous cell carcinoma (14). The aberrant expression of NOB1 was also found in breast-infiltrating ductal carcinoma and was possibly involved in tumorigenesis and development (15). Expression of NOB1 mRNA and protein in papillary thyroid carcinoma tissue was significantly higher than in normal and benign thyroid tissue (16). There were also significant associations between NOB1 expression and TNM stage, lymph node metastasis and histopathological grade of non-small cell lung cancer (NSCLC) and prostate carcinoma (17,18). Knockdown of NOB1 decreased the proliferation of several types of human cancer cells, including breast and ovarian cancer, hepatocellular carcinoma, gliomas, osteosarcoma, NSCLC and colon cancer cells (19-25). However, the roles of NOB1 in OSCC have not been reported.

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To determine the potential role of NOB1 in OSCC, an immunohistochemical analysis of NOB1 protein in OSCC tumors was performed. A loss-of-function analysis was then performed by applying a NOB1 short hairpin RNA (shNOB1)-expressing lentivirus (Lv-shNOB1) to two OSCC cell lines, CAL27 and TCA-8113. The effect of NOB1 knockdown on OSCC cell proliferation, colony formation, apoptosis and cell cycle progression was investigated.

Materials and methods

Cell lines, reagents and antibodies. Human OSCC CAL27 and TCA-8113 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (CAL27) or RPMI-1640 medium (TCA-8113) containing 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37˚C with 5% CO_2 and 95% humidity. Rabbit anti-NOB1 polyclonal antibody used for western blot analysis was purchased from Novus Biologicals (Littleton, CO, USA). Anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Immunohistochemical assay reagents were purchased from Abcam (Boston, MA, USA) and Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Anti-GAPDH mAb was purchased from Santa Cruz Biotechnology, Inc. The Annexin V-APC apoptosis detection kit was purchased from eBioscience (San Diego, CA, USA). Propidium iodide (PI) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Immunohistochemical staining. Immunohistochemical analysis was first performed on tissue chip. The chip used in the present study was no. OR601a, which was purchased from US Biomax, Inc. (Rockville, MD, USA). The patient information is available at the link: http://www.biomax.us/tissue-arrays/Oral_Cavity/OR601a (Table I).

Freshly prepared immunohistochemical staining was carried out. Briefly, tissue sections (5-μm) were dewaxed, followed by quenching the endogenous peroxidase with 3% H_2O_2 in methanol for 30 min. Prior to staining, non-specific binding was blocked by incubation with 10% BSA in phosphate-buffered saline (PBS) at 37˚C for 1 h. Tissue sections were incubated with specific antibodies in PBS containing 1% BSA at 4˚C overnight, followed by incubation with a horseradish peroxidase-conjugated anti-mouse or rabbit antibody. Color was then developed by incubation with an ImmunoPure Metal Enhanced Diaminobenzidine (DAB) Substrate kit (Pierce, Rockford, IL, USA). After each incubation, the tissue sections were washed three times with PBS for 10 min, and then counterstained with hematoxylin. For determination of NOB1, cytosolic and nuclear staining of yellowish or brownish granules was graded as: 0 for background, 1 for faint, 2 for moderate and 3 for strong staining. In addition, positive staining areas in the entire tissue section were graded as: 0 for <5%, 1 for 5-25%, 2 for 26-50%, 3 for 51-75% and 4 for 76-100%. Combining these two parameters, 0-2 and ≥3 were considered negative and positive stainings, respectively.

Lentivirus-mediated shRNA delivery. Sequences of NOB1 shRNA were inserted into the pGCSIL-GFP lentivirus RNAi expression system (GeneChem, Shanghai, China). The shRNA-containing vectors were co-transfected together into 293T cells with pHelper1.0 and lentiviral helper plasmid pHelper2.0 using Lipofectamine 2000 (Invitrogen-Life Technologies, Carlsbad, CA, USA) to generate the respective lentiviruses. Viral stocks collected from 293T cells were used to infect CAL27 and TCA-8113 cells three days after infection. The target sequence of NOB1 siRNA was: GGTTAA GGTGAGCTCATCG. The negative control scramble sequence of siRNA was: TTCTCCGAACGTGTCACGT, which does not target any genes in humans, mice or rats as determined by screening with NCBI RefSeq. The stem-loop-stem oligos [short-hairpin RNAs (shRNAs)] were produced, annealed and ligated into the *Age*I/*Eco*RI-linearized pGV112 vector (GeneChem). The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing. The mRNA and protein levels were measured 72 h after lentivirus infection.

RT-PCR analysis. Total RNA was isolated using TRIzol reagent (Invitrogen-Life Technologies) and reverse transcribed using a PrimeScript® RT reagent kit (Takara, Dalian, China). cDNA was normalized using GAPDH. RT-PCR was performed by three-step methods using a SYBR® Premix *Ex* Taq™ II kit (Takara) with 55˚C annealing temperature and 40 amplification cycles. The individual test was carried out in triplicate. GAPDH was used as an internal control. The relative amount of each cDNA was analyzed by means of $2^{-\Delta\Delta Ct}$. Primers for qPCR for NOB1 and GAPDH were as follows: NOB1-F, ATCTGCCCTACAAGCCTAAAC and -R, TCCTCCTCC TCCTCCTCAC; and GAPDH-F, TGACTTCAACAG CGACACCCA and -R, CACCCTGTTGCTGTAGCCAAA.

Western blotting. Protein samples prepared from CAL27 and TCA-8113 cells five days after NOB1 shRNA lentivirus or control shRNA lentivirus infection were subjected to 10% SDS-PAGE (20 μg protein each lane), transferred to PVDF membranes (Millipore, Kankakee, IL, USA) and detected with rabbit anti-NOB1 or rabbit anti-GAPDH antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. GAPDH was used as an internal control. Proteins were then detected using an ECL kit (Amersham, Piscataway, NJ, USA) and exposed to X-ray film. Bands on the X-ray film were quantified with an ImageQuant densitometric scanner (Molecular Dynamics, Sunnyvale, CA, USA).

Cell proliferation assay. Five days after lentivirus infection, CAL27 or TCA-8113 cells were trypsinized, resuspended, seeded in a 96-well plate with a density of $2x10^3$ cells/well and incubated at 37˚C. The number of viable cells was measured at daily intervals (days 1-5). At each time-point, 20 μl of 5 mg/ml MTT (Dingguo Biotechnology, Beijing, China) was added and incubation was continued for 4 h. At the end of the incubation period, the medium was removed carefully and 150 μl of acidified isopropanol (in 0.01 M HCl) was added. The plates were agitated and the absorbance was measured at 490 nm on the spectrophotometer Biotek ELx800 (Beijing, China). Each data point was collected from five parallel wells.

Colony formation assay. The CAL27 and TCA-8113 cells were seeded in 6-well plates ($8x10^2$ cells/well) (in three

Table I. Patient information of OSCC tissue chip OR601a.

No.	Gender	Age (years)	Organ	Pathology diagnosis	Grade	Stage	TNM	Type
1	M	78	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
2	F	51	Tongue	Squamous cell carcinoma	1	Iva	T4N0M0	Malignant
3	F	75	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
4	M	69	Tongue	Squamous cell carcinoma	1	III	T3N0M0	Malignant
5	F	56	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
6	F	35	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
7	F	39	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
8	M	64	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
9	M	63	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
10	F	77	Tongue	Squamous cell carcinoma	2	I	T1N0M0	Malignant
11	F	41	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
12	M	53	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
13	M	50	Tongue	Squamous cell carcinoma	1	III	T3N0M0	Malignant
14	F	36	Tongue	Squamous cell carcinoma	2	I	T1N0M0	Malignant
15	M	58	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
16	F	63	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
17	F	55	Tongue	Squamous cell carcinoma	2	II	T2N0M0	Malignant
18	M	76	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
19	F	50	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
20	M	44	Tongue	Squamous cell carcinoma	1	III	T2N1M0	Malignant
21	F	53	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
22	F	67	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
23	M	60	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
24	M	55	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
25	M	61	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
26	M	55	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
27	M	59	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
28	F	46	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
29	F	45	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
30	M	61	Tongue	Squamous cell carcinoma (fibrous tissue and blood vessel)	-	II	T2N0M0	Malignant
31	F	48	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
32	F	52	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
33	M	64	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
34	F	46	Tongue	Squamous cell carcinoma (sparse)	1	II	T2N0M0	Malignant
35	F	48	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
36	M	80	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
37	M	49	Tongue	Squamous cell carcinoma (fibrous tissue and skeletal muscle)	-	I	T1N0M0	Malignant
38	M	60	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
39	M	57	Tongue	Squamous cell carcinoma	1	I	T1N0M0	Malignant
40	M	45	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
41	F	47	Tongue	Squamous cell carcinoma	1	II	T2N0M0	Malignant
42	M	37	Tongue	Squamous cell carcinoma	1	III	T2N1M0	Malignant
43	M	60	Tongue	Squamous cell carcinoma	2	II	T2N0M0	Malignant
44	F	40	Tongue	Squamous cell carcinoma	3	II	T2N0M0	Malignant
45	M	49	Tongue	Squamous cell carcinoma	1-2	I	T1N0M0	Malignant
46	M	50	Tongue	Squamous cell carcinoma	3	II	T2N0M0	Malignant
47	M	60	Tongue	Squamous cell carcinoma	3	I	T1N0M0	Malignant
48	F	56	Tongue	Squamous cell carcinoma	3	II	T2N0M0	Malignant
49	M	77	Tongue	Squamous cell carcinoma	3	II	T2N0M0	Malignant

Table I. Continued.

No.	Gender	Age (years)	Organ	Pathology diagnosis	Grade	Stage	TNM	Type
50	M	56	Tongue	Squamous cell carcinoma	2	III	T2N1M0	Malignant
51	M	76	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
51	M	76	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
52	M	38	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
53	F	51	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
54	M	30	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
55	M	50	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
56	M	2 months	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
57	M	49	Tongue	Cancer adjacent normal tongue tissue	-	-	-	NAT
58	M	40	Tongue	Cancer adjacent normal gingiva tissue	-	-	-	NAT
59	M	25	Tongue	Cancer adjacent normal gingiva tissue	-	-	-	NAT
60	M	62	Tongue	Cancer adjacent normal mucous membrane tissue of pars palatalis	-	-	-	NAT
61	M	42	Adrenal gland	Pheochromocytoma (tissue marker)	-			Malignant

OSCC, oral squamous cell carcinoma; M, male; F, female; TNM, tumor node metastasis; NAT, not applicable type.

duplicate wells) and cultured at 37˚C in 5% CO_2. After two weeks, the cells were washed with PBS once and fixed with paraformaldehyde for 30 min and washed with PBS and stained with Giemsa for 20 min. ddH_2O was used to wash the cells three times to obtain a clean background. The number of colonies and cell number in each colony were counted and statistically analyzed.

Flow cytometric analysis. Apoptosis assay was carried out with Annexin V-APC staining. Cells were harvested by centrifugation at 1,200 rpm for 5 min after six days of infection. The pellets were washed twice with cold PBS, fixed with chilled 70% ethanol, centrifuged at 1,500 rpm for 5 min to discard ethanol and resuspended with PBS sequentially. Suspensions were filtrated through 400-mesh membrane and centrifuged at 1,200 rpm for 5 min. The cells were resuspended with 1X Annexin V staining buffer and stained with Annexin V-APC at room temperature for 15 min in the dark for the flow cytometric analysis. Each experiment was carried out in triplicate.

The cell cycle distribution was analyzed with PI staining. Briefly, $1.5x10^5$ cells that were infected with the lentivirus constructs for 4 days were seeded in 6-cm dishes and cultured for 40 h at 37˚C. The cells were harvested, washed with PBS and fixed with 70% cold ethanol. The cells were then collected by centrifugation, resuspended in PBS containing 100 μg/ml of DNase-free RNase and 40 μg/ml PI, and incubated for 1 h at 37˚C. A total of $1.0x10^4$ fixed cells were analyzed by FACS (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. One-way ANOVA and the Student's t-test were used for raw data analysis. Statistical analysis was performed using SPSS 12.0 software package. Values in the text and figures are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant result.

Results

Expression of NOB1 in OSCC tissue. Immunohistochemical analysis was performed on tissue chip with 50 OSCC cancer tissues and 10 cancer adjacent normal tongue tissues from patients with medical records (no. OR601a; US Biomax, Inc.). NOB1-positive stainings in different genders, ages, TNM and pathological grades were calculated (Table II). NOB1 expression in OSCC cancers had no statistical significance in patients with different genders and ages. By contrast the NOB1-positive rate in TNM grades II, III and IV (88.24%) was

Table II. Association between NOB1 expression and pathological parameters in tissue chip.

Parameters	N	NOB1			χ^2	P-value
		+	-	(%)		
Gender					0.148	0.7
Male	28	18	10	64.28		
Female	22	14	8	63.63		
Age (years)					2.002	0.157
≥50	34	24	10	70.59		
<50	16	8	8	50.00		
TNM					6.566	0.01
I	33	17	16	51.52		
II, III, IV	17	15	2	88.24		
Pathological grades					4.93	0.026
I	31	16	15	51.61		
II, III	19	16	3	84.21		

NOB1, NIN1/RPN12 binding protein 1 homolog. TNM, tumor node metastasis.

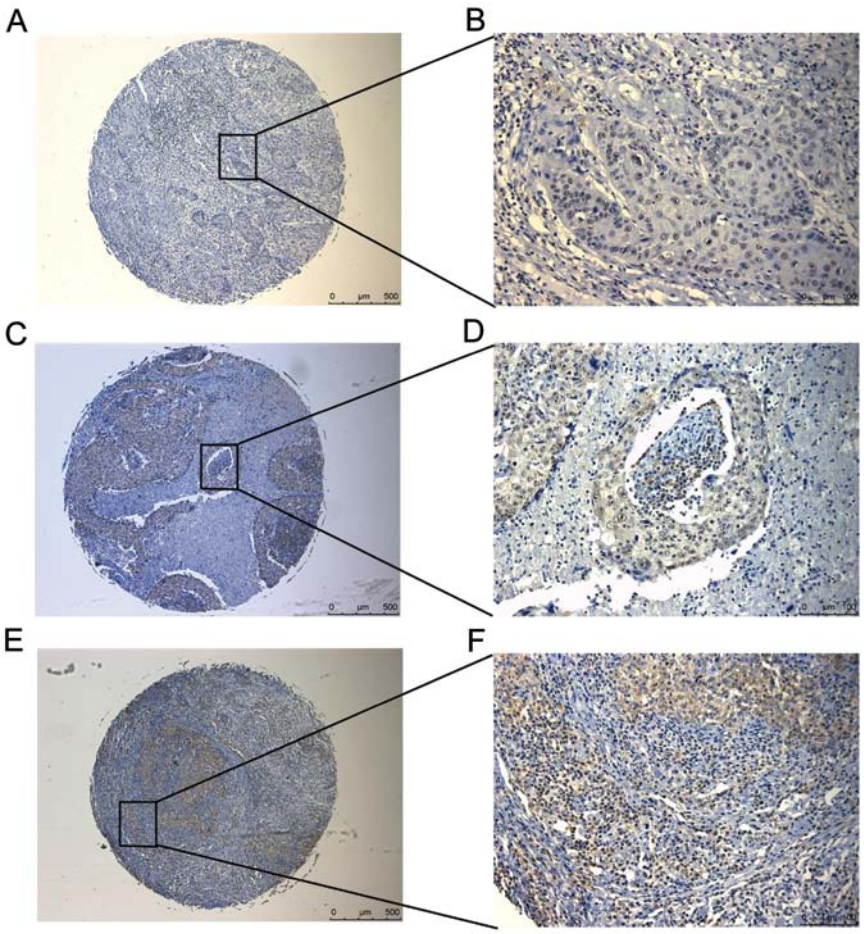

Figure 1. Typical immunohistochemical staining of NOB1 in malignant OSCC biopsy specimens of different WHO grades. Specimens in A, C and E represent grades I, II and III OSCC (magnification, x200). Specimens in B, D and F represent grade I, II and III OSCC (magnification, x400) compared to A, C and E, respectively. Brown staining indicated NOB1-positive staining.

significantly higher than grade I (51.52%, P=0.01). In addition, the NOB1-positive rate in pathological grades II and III (84.21%) was significantly higher than grade I (51.61%, P=0.026). The same result was observed in immunohistochemical staining

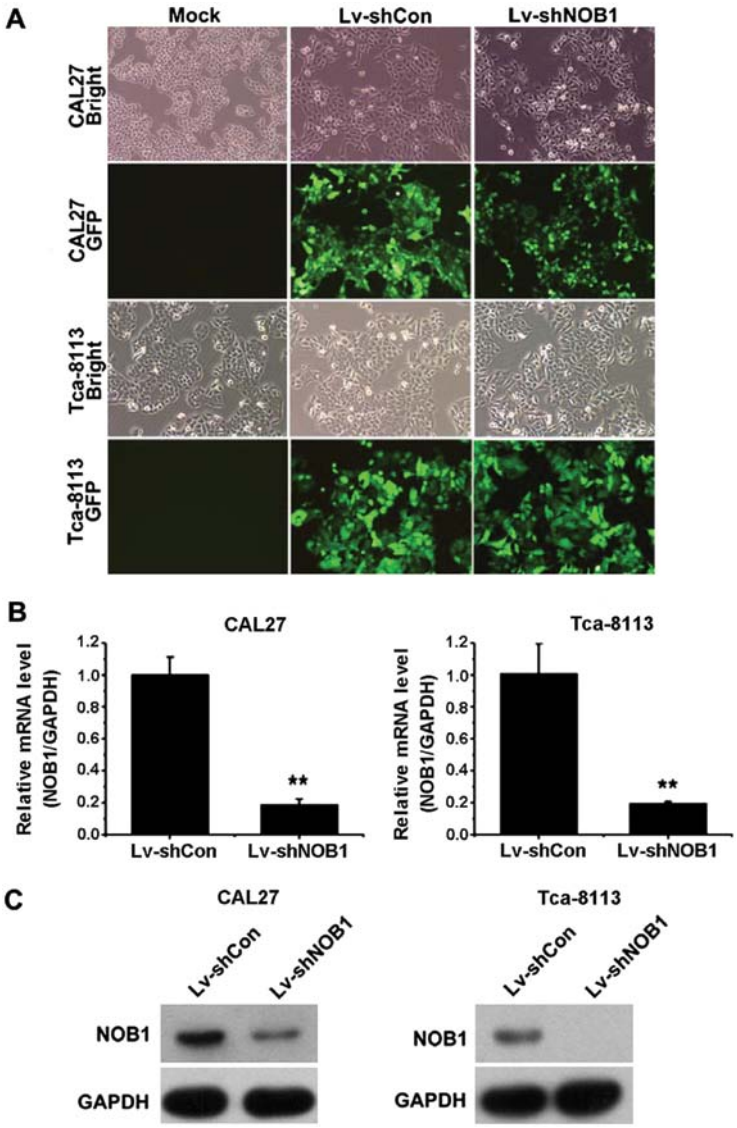

Figure 2. Lentivirus-shRNA mediated knockdown of NOB1 in glioma cells. (A) Infection efficiency of lentivirus in human OSCC CAL27 and TCA-8113 cells. Representative images of CAL27 and TCA-8113 cells after 72 h of lentivirus infection in bright and fluorescence are shown (magnification, x100). (B) Expression analyses of NOB1 mRNA levels by quantitative PCR (qPCR) analysis. (C) Expression analyses of NOB1 protein levels by western blot analysis. (C) Expression analyses of NOB1 protein levels by western blot analysis. Con, uninfected cells; Lv-shCon, cells infected with Lv-shCon lentivirus; Lv-shNOB1, cells infected with Lv-shNOB1 lentivirus. GAPDH gene and protein were used as internal controls for qPCR and western blot analysis, respectively. **P<0.01.

of OSCC tissue sections. As shown in Fig. 1, NOB1-positive staining increased with the pathological grades of OSCC cancer. Thus, elevation of NOB1 expression plays an important role in the pathogenesis of human OSCC.

Lentivirus-mediated shRNA inhibited NOB1 mRNA and protein expression in CAL27 and TCA-8113 cells. Approximately half of intraoral squamous cell carcinomas begin on the floor of the mouth or on the lateral and ventral surfaces of the tongue (2). Thus, to investigate the role of NOB1 in OSCC, a human OSCC cell line, CAL27, and a human tongue squamous cell carcinoma cell line, TCA-8113, were selected to study *in vitro*. A loss-of-function study was carried out by lentivirus-mediated shRNA knockdown of NOB1. The lentiviral vector system was constructed to express a shRNA targeting NOB1 and GFP as a reporter gene. To determine the infection efficiency of lentivirus of CAL27 and TCA-8113 cells, at 30% cell density, the cells infected with Lv-shNOB1 and Lv-shCon vectors were observed under a fluorescence microscope after three days of infection. As shown in Fig. 2A, >90% of CAL27 and TCA-8113 expressed GFP, which indicated a high efficiency infection of the lentivirus.

To verify the knockdown efficiency of Lv-shNOB1, the mRNA and protein expression of NOB1 in CAL27 and TCA-8113 was detected by qPCR and western blot analysis five days after lentivirus infection. As shown in Fig. 2B, the mRNA expression levels of NOB1 in CAL27 and TCA-8113 infected by Lv-shNOB1 were downregulated by 80.8% (P<0.05) and 80.2% (P<0.05) compared to the Lv-shCon infection group, respectively. As shown in Fig. 2C, the protein expression of NOB1 in CAL27 and TCA-8113 cells infected with Lv-shNOB1 was also significantly decreased compared to the Lv-shCon infection group.

Knockdown of NOB1 significantly inhibited OSCC cell proliferation. To investigate the effect of NOB1 knockdown on

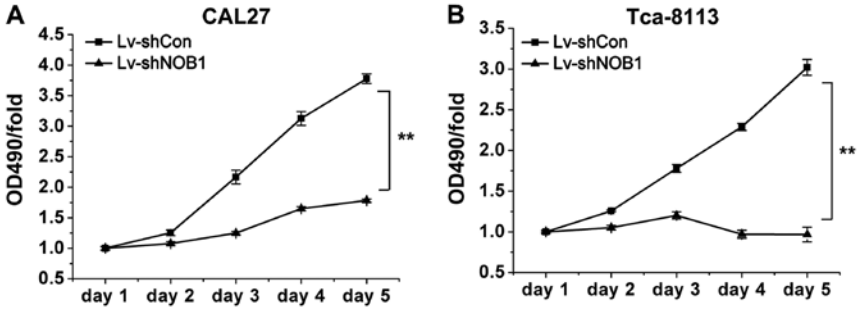

Figure 3. *In vitro* proliferation assays of CAL27 (A) and TCA-8113 (B). Mock, uninfected cells; Lv-shCon, cells infected with Lv-shCon lentivirus; Lv-shNOB1, cells infected with Lv-shNOB1 lentivirus. Cell proliferation in Lv-shNOB1 groups is significantly inhibited compared to Lv-shCon as demonstrated by MTT assay.

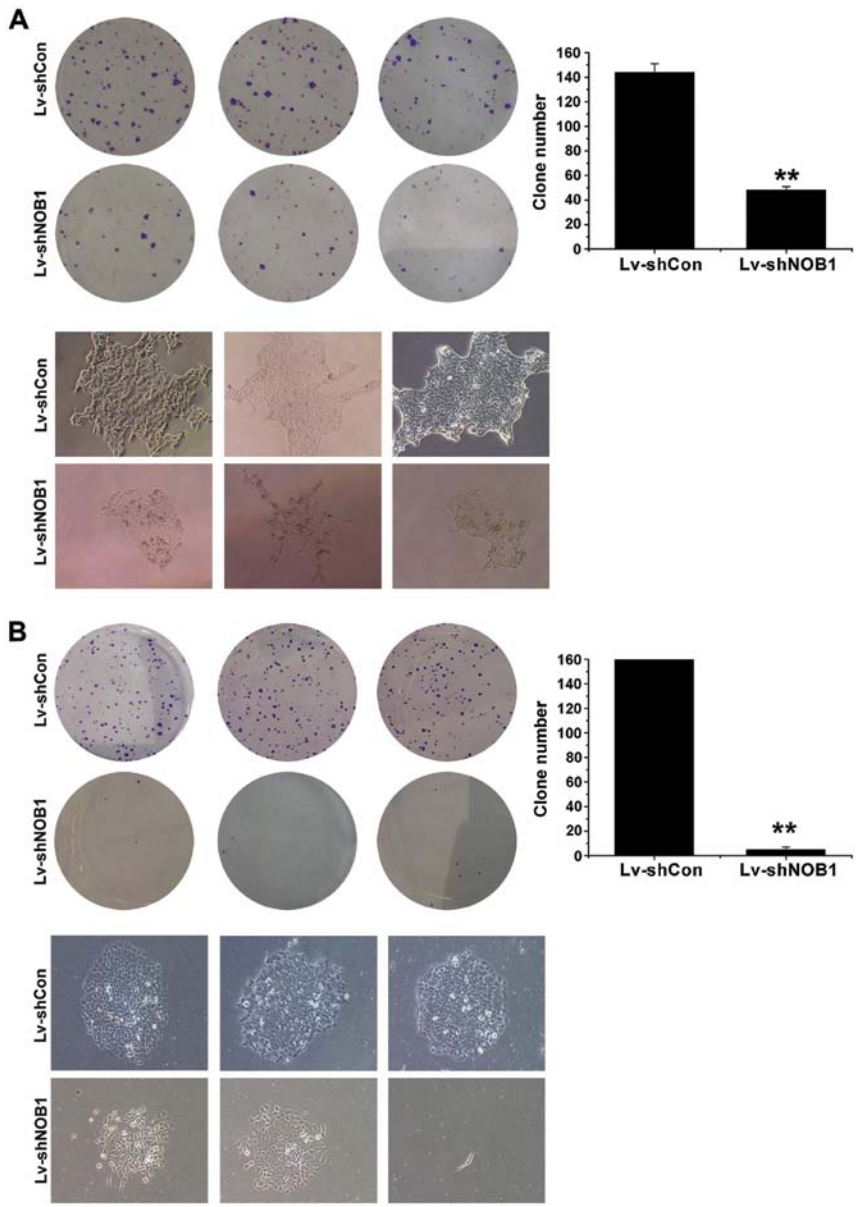

Figure 4. Clone formation of (A) CAL27 and (B) TCA-8113. Cells were allowed to grow into natural colonies in a 6-well plate. After staining with Giemsa, the number of colonies was counted in each groups. Three representative colony formation wells of each group are shown in the upper left of A and B. The colony number is shown in the upper right of A and B. The lower left of A and B show three representative colonies of each group. Con, uninfected cells; Lv-shCon, cells infected with Lv-shCon lentivirus; Lv-shNOB1, cells infected with Lv-shNOB1 lentivirus. $^{**}P<0.01$.

cell proliferation, an MTT assay was performed in CAL27 and TCA-8113 cell lines five days after lentivirus infection. Data were collected from five parallel wells in each group for 5 days consecutively. As shown in Fig. 3, lentivirus-mediated shRNA

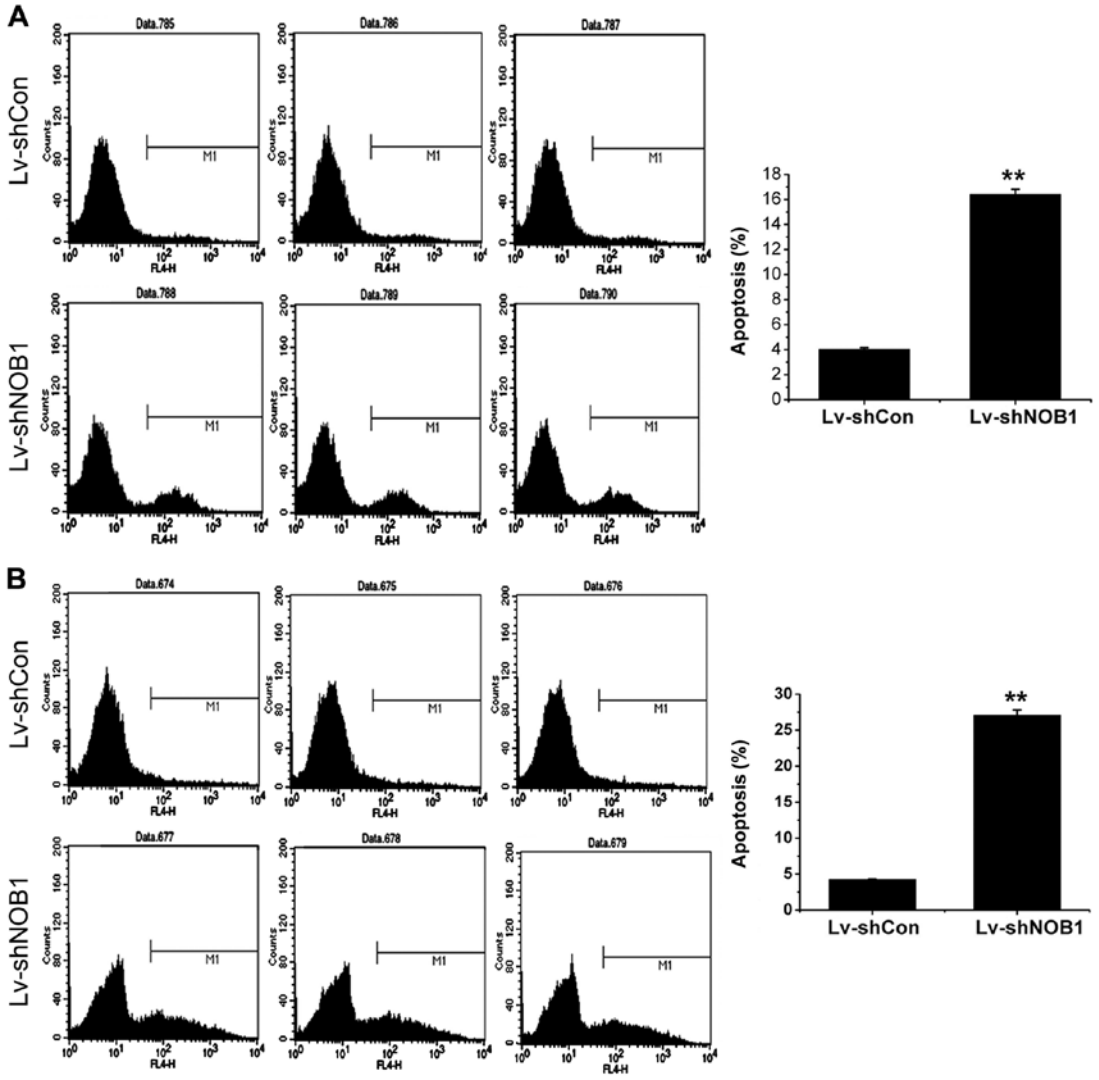

Figure 5. Knockdown of NOB1 increases apoptosis of OSCC (A) CAL27 and (B) TCA-8113 cells *in vitro*. For CAL27 and TCA-8113 cells, Lv-shNOB1 knockdown showed a significant increase in apoptosis compared to lentivirus control shRNA, Lv-shCon, infection. **P<0.01, significant difference from the Lv-shCon group.

knockdown of NOB1 significantly inhibited the proliferation of CAL27 (Fig. 3A) and TCA-8113 (Fig. 3B) compared to cells infected with the control lentivirus. On day 5 of the assay, Lv-shRNA infection inhibited the proliferation of CAL27 by 52.8% (P<0.01) and TCA-8113 by 67.9% (P<0.01). Compared to the mock group (CAL27 and TCA-8113 cells without lentivirus infection), the cell number in Lv-shCon was slightly decreased, which indicated that lentivirus infection had a slight effect on the proliferation of CAL27 and TCA-8113. The result indicated that NOB1 played an important role in the proliferation of CAL27 and TCA-8113 cells.

Knockdown of NOB1 significantly inhibited the colony-forming ability of OSCC cells. To study the long-term effect of NOB1 shRNA lentivirus on the cell growth, colony-forming experiments were performed on CAL27 and TCA-8113 cell lines. After 72 h of lentivirus infection, the cells were allowed to grow for 11 days with media replacement every two days to form colonies. As shown in Fig. 4A upper left, the number of colonies in Lv-shNOB1-treated CAL27 were significantly less than that in the Lv-shCon-treated group. As shown in Fig. 4A upper right, the colony number was 48±3 in Lv-shNOB1 compared to 144±7 in the Lv-shCon group (P<0.01). The sizes of colonies in Lv-shNOB1 were significantly smaller than that in the Lv-shCon group (as shown in Fig. 4A lower left). As shown in Fig. 4B upper left, the number of colonies in Lv-shNOB1-treated TCA-8113 were significantly less than that in the Lv-shCon-treated group. As shown in Fig. 4B upper right, the colony number was 5±2 in Lv-shNOB1 compared to 206±6 in the Lv-shCon group (P<0.01). The sizes of the colonies in Lv-shNOB1 were significantly smaller than that in the Lv-shCon group (as shown in Fig. 4B lower left). Our results indicated that the downregulation of NOB1 significantly decreased the colony formation of OSCC cells *in vitro*.

Knockdown of NOB1 significantly increased apoptosis of OSCC cells. To detect the effect of NOB1 knockdown in OSCC cells, Annexin V staining and flow cytometric analysis were carried out on CAL27 and TCA-8113 after six days of lentivirus infection when cell confluency reached 90%. As shown in Fig. 5A, the apoptotic rate of CAL27 cells in the

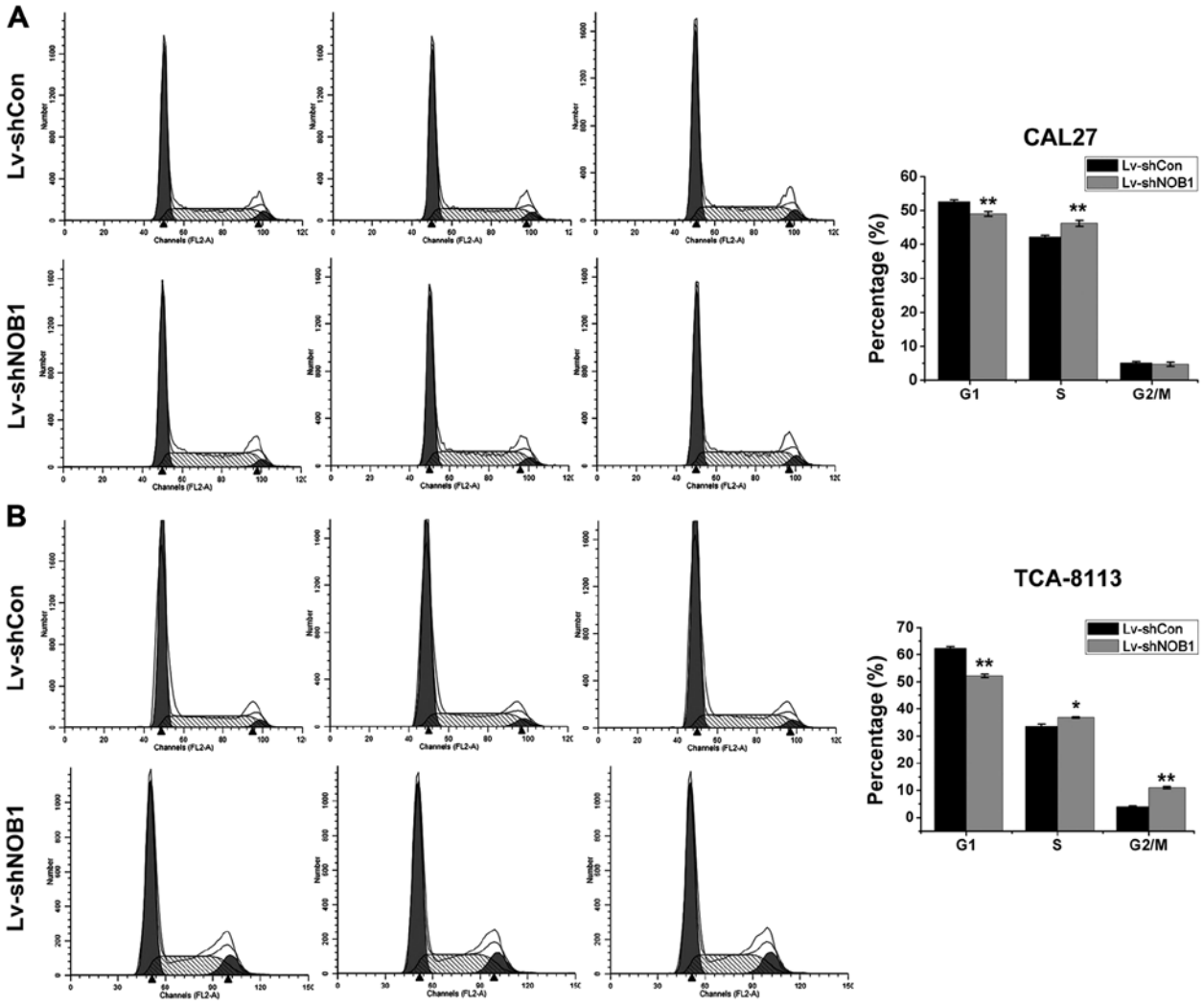

Figure 6. Knockdown of NOB1 decreases the ratio of G1/S phase cells in OSCC (A) CAL27 and (B) TCA-8113 cells. The proportion of different cell cycle phases was quantified by PI staining followed by flow cytometric analysis. Con, uninfected cells; Lv-shCon, cells infected with Lv-shCon lentivirus; Lv-shNOB1, cells infected with Lv-shNOB1 lentivirus. *P<0.05, **P<0.01.

Lv-shNOB1 group was ~16.40 compared to 4.04% in the Lv-shCon group (P<0.05). As shown in Fig. 5B, the apoptotic rate of TCA-8113 cells in the Lv-shNOB1 group was ~27.10 compared to 4.27% in the Lv-shCon group (P<0.05).

Effects of NOB1 knockdown on cell cycle distribution. To elucidate the impact of Lv-shNOB1 knockdown of NOB1 on the cell cycle progression of OSCC cells, CAL27 and TCA-8113 cell lines were subjected to a PI staining flow cytometric assay six days after lentivirus infection. The Lv-shNOB1-infected CAL27 and TCA-8113 cells exhibited a decreasing portion of cells in the G1 phase (P<0.01, Fig. 6A and B) and an increasing portion of cells in the S phase (p<0.01 for CAL27 Fig. 6A, and p<0.05 for TCA-8113 Fig. 6B), compared to the cells infected with Lv-shCon. Of note, the Lv-shNOB1 knockdown of NOB1 also increased the portion of G2/M phase cells in TCA-8113 but not in CAL27, suggesting that NOB1 showed various function in cell cycle in different cell lines. The results indicated that the knockdown of NOB1 induced the S-phase arrest in OSCC cells and induced G2/M-phase arrest in the OSCC cell lines.

Discussion

OSCC, one of the most common types of cancer usually found in smoking and heavy-drinking middle-aged men, is on the increase in non-smoking and non-heavily-drinking young individuals, especially, young men. This phenomenon may be partially attributed to HPV infection from oral sexual behavior (3,26). OSCC is becoming an important cause of morbidity and mortality, especially in developing countries and its prevalence may rise in the foreseeable future. Therefore, the study on the mechanism of OSCC genesis and development, especially, the mechanism of how the virus involves, is of great importance.

In normal cells, NOB1 was found to be involved in two key cell processes. Firstly, NOB1 facilitates the maturation of the 20S proteasome and is then degraded by 26S proteasome to complete 26S proteasome biogenesis, which means NOB1 is important in ubiquitin-mediated protein degradation (5). Secondly, NOB1 accompanies the pre-40S ribosomes during nuclear export and is cleaved at the D-site of 20S pre-rRNA to form mature 18S rRNA, which means NOB1 is also involved

in protein synthesis (6-8). The two functions of NOB1 indicate that NOB1 is important in protein homeostasis. Abnormal regulation of the expression of NOB1 in cells may lead to dysfunction of the protein synthesis and protein degradation and disturbs the protein homeostasis. Rapidly growing cancer cells require more proteins for DNA replication and cell division. Therefore, it is not surprising to identify NOB1 upregulation in cancer. In addition, NOB1 was found to be a target of microRNA-326 and may increase the proliferation of glioma cells by activating the MAPK pathway by increasing the phosphorylation of ERK1/2, JNK and p38 (27). Therefore, in cancer cells, the protein homeostasis and signal transduction function of NOB1 were modulated to facilitate the proliferation and development of cancers.

In the present study, NOB1 was highly expressed in OSCC cancers in clinical specimens. Expression of NOB1 increased with the pathological grades of OSCC, which indicated that NOB1 may be involved in the development of OSCC. The hypothesis was tested with loss-of-function of NOB1 in two OSCC cell lines. In the cell assay, NOB1 knockdown in OSCC cells decreased cell proliferation and colony formation, increased cell apoptosis and induced cell cycle arrest in S phase. The results were confirmed by the knockdown of NOB1 in normal cells HEK293 (data not shown). Knockdown of NOB1 in HEK293 did not show any significant difference in cell proliferation, colony formation and apoptosis, which means NOB1 is not a housekeeping gene in cells. Expression of NOB1 was specifically increased in OSCC cancers but not in normal cells.

It was reported that NOB1 is also involved in the MAPK pathway (27). The MAPK pathway plays a central role in tumorigenesis and tumor development (28). The MAPK pathway is also reported to mediate the insulin-like growth factor (IGF) signaling to promote the proliferation of OSCC. In view of the importance of MAPK in cell proliferation and differentiation, many viruses hijack the MAPK pathway in the progress of virus induced tumor genesis. MAPK was reported to participate in human papillomavirus type (HPV)-induced human cervical squamous carcinoma (29,30). It is hypothesized that HPV infection is one of the histopathologic risk factors in young OSCC patients (1,31). However, the mechanism of HPV infection on the tumorigenesis and tumor development of OSCC remains to be elucidated. Based on the above information, we hypothesize that NOB1 is involved in the malignant transformation of oral squamous cell after HPV infection.

In conclusion, we have shown that the expression level of NOB1 may be an indicator of the aggressiveness of OSCC. A lentivirus-mediated siRNA study in CAL27 and TCA-8113 revealed that NOB1 plays an important role in proliferation and anti-apoptosis in OSCC cells. NOB1 may be involved in the malignant transformation of oral squamous cells after HPV infection. Notably, the mechanism of how NOB1 exerts its effect in OSCC cells remains to be determined.

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