SNHG20 serves as a predictor for prognosis and promotes cell growth in oral squamous cell carcinoma

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Abstract. Accumulating evidence indicates that long non-coding RNAs (lncRNAs) serve important roles in various tumor types, including colorectal cancer and gastric cancer. The present study aimed to investigate the contribution of the lncRNA small nucleolar RNA host gene 20 (SNHG20) in oral squamous cell carcinoma (OSCC) progression. It was demonstrated that SNHG20 expression was significantly increased in OSCC tissue specimens, compared with in adjacent non-tumor tissue specimens. The increased SNHG20 expression in OSCC tissue specimens was associated with tumor differentiation and Tumor-Node-Metastasis stage. Kaplan-Meier analysis and log-rank tests indicated that Higher SNHG20 expression predicted a poor overall survival (OS) rate in patients with OSCC. Multivariate Cox proportional hazards regression analysis demonstrated that increased SNHG20 expression was an independent predictor for the OS of patients with OSCC. Knockdown of SNHG20 expression in OSCC cells suppressed proliferation. The cell proliferation-associated proteins proliferating cell nuclear antigen and Ki67 expression levels were reduced when SNHG20 was knocked down in OSCC cells; thus, the results indicated that SHNG20 may serve as a predictor and potential target for OSCC treatment.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent tumor types, with >500,000 newly diagnosed cases reported globally in 2008 (1,2). Oral squamous cell carcinoma (OSCC) is the most frequent subset of HNSCC worldwide (2). Despite large advances in diagnosis and therapy for the disease, the overall 5-year survival rate of patients with advanced stage OSCC remains <50% (3,4); thus, it is important to investigate effective diagnostic biomarkers and therapeutic targets for improving the prognosis of patients with OSCC.

Long non-coding RNAs (lncRNAs), a class of non-protein coding transcripts >200 nucleotides, have been identified as vital regulators for OSCC progression (5,6). For example, the lncRNA colon cancer-associated transcript 1 (CCAT1) is overexpressed in OSCCs and a higher CCAT1 expression in OSCCs has been reported to predict poor prognosis (7). Additionally, the lncRNA urothelial cancer-associated 1 (UCA1) contributes to the progression of OSCC by regulating the WNT/β-catenin signaling pathway (8). Furthermore, the lncRNA metastasis-associated lung adenocarcinoma transcript 1 promotes tumor growth and metastasis by inducing epithelial-mesenchymal transition in OSCC (9). The lncRNA UCA1 promotes proliferation and cisplatin resistance of OSCC by suppressing microRNA-184 (miR-184) expression (10). These aforementioned studies indicated that lncRNAs were involved in OSCC progression.

Small nucleolar RNA host gene 20 (SNHG20), localized at 17q25.2, has been determined to be involved in a number of tumor types, including colorectal cancer and gastric cancer. SNHG20 expression is significantly upregulated in colorectal cancer and increases cell proliferation (11). Additionally, SNHG20 promotes gastric cancer progression by inhibiting p21 expression and regulating the glycogen synthase kinase-3β/β-catenin signaling pathway (12); however, the role of SNHG20 in OSCC remains unknown.

In the present study, it was determined that SNHG20 was increased in OSCC tissues, compared with in adjacent non-tumor tissues. Higher SNHG20 expression predicted a poor overall survival rate of patients with OSCC. Furthermore, knockdown of SNHG20 in OSCC cells suppressed proliferation ability; thus, the results demonstrated that SHNG20 may serve as a predictor and potential target for OSCC treatment.

Materials and methods

Clinical tissue specimens. Human OSCC tissue specimens and matched adjacent non-tumor tissue specimens were obtained from 40 patients at the Department of Stomatology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, China) between April 2008 and July 2013. Following resection, tissue specimens were immediately placed in RNAlater® solution (Qiagen GmbH, Hilden, Germany) and were then stored in liquid nitrogen for further

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analysis. The clinicopathological factors of patients are summarized in Table I. All patients were clinically examined and staged according to the Tumor-Node-Metastasis (TNM) and Union for International Cancer Control classifications (13). The follow-up duration is the period from surgery to the last follow-up date. The protocol of the present study was approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine, and written informed consent was obtained from all patients.

Cell lines culture. A total of three human OSCC cell lines (SCC4, HN4 and SCC15) and the normal human oral keratinocyte (NHOK) cell line were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA). All cells were cultured at 37˚C in a humidified air atmosphere containing 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues and cell lines, including HN4SCC4, HN4, SCC15 and NHOK cells with the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cDNA was produced by reverse transcribing 100 ng total RNA. Reverse transcription assay was conducted to obtain the complementary DNA (cDNA) by Prime Script RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocols. The relative expression of SNHG20 was detected with the SYBR® Green Master Mix (Takara Bio, Inc., Otsu, Japan) using a Step One Plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used as the endogenous control. The relative expression quantification of SNHG20 was calculated using $2^{-\Delta\Delta Cq}$ method (14). The thermocycling conditions for qPCR were as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 12 sec and 58˚C for 40 sec. The primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The primer sequences were as follows: SNHG20, forward, 5'-ATGGCTATAAATAGATACACG C-3', and reverse, 5'-GGTACAAACAGGGAGGGA-3'; and GAPDH, forward, 5'-ACAGTCAGCCGCATCTTCT-3' and reverse, 5'-GACAAGCTTCCCGTTCTCAG-3'.

Cells transfection. A total of two small interfering RNAs (siRNAs) against SNHG20 were purchased from Shanghai GenePharma Co., Ltd. The siRNAs sequences were as follows: si-NC, 5'-GGATACGGAGTACTATAGC-3'; si-SNHG20-1, 5'-GCCUAGGAUCAUCCAGGUUTT-3'; and si-SNHG20-2, 5'-GCCACUCACAAGAGUGUAUTT-3'. When cell confluence reached 80-90%, 100 nm si-negative control (si-NC), si-SNHG20-1 or si-SNHG20-2 were transfected into SCC4 or SCC15 cells using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were harvested at 48 h after transfection.

Cell proliferation assay. Cell proliferation capacity was detected with a Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Nantong, China). Subsequently, SCC4 and SCC15 cells were seeded into 96-well plates ($2x10^3$ cells/well), and transfected with si-NC, si-SNHG20-1 or si-SNHG20-2. At 1, 2, 3 and 4 days after cell transfection, cells were stained using 10 μl CCK-8 reagent. After 2 h of incubation at 37˚C, cell proliferation capacity was measured using a microplate reader (Enspire 2300 Maltilabel Reader; PerkinElmer, Inc., Waltham, MA, USA), and the absorbance of samples was measured at 450 nm.

Colony formation assay. The transfected cells from each group were seeded into 12-well plates ($5x10^2$ cells/well) and cultured for 14 days at 37˚C in a humidified air atmosphere containing 5% CO_2. Subsequently, the colonies were then fixed with 100% methanol at room temperature for 20 min and stained with 0.1% crystal violet at room temperature for 20 min. Finally, cells were counted and imaged under a X71 fluorescence microscope (magnification, x200; Olympus Corporation, Tokyo, Japan).

Western blot analysis. SCC4 and SCC15 cells were lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) and quantified using a bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocols. A. total of 20 μg total protein lysates were separated on 10% SDS-PAGE electrophoresis and then were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked using 5% free-fat milk for 1 h at room temperature and then were incubated with anti-proliferating cell nuclear antigen (PCNA) (cat. no. ab92552; dilution, 1:3,000; Abcam, Cambridge, UK), anti-Ki67 (cat. no. ab15580; dilution, 1:3,000; Abcam) and anti-GAPDH (cat. no. sc-20358, dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, CA, USA) antibodies at 4˚C overnight. Subsequently, membranes were incubated with secondary antibody anti-mouse IgG conjugated with horseradish peroxidase (dilution, 1:5,000; cat. no. ab97040; Abcam) for 2 h at room temperature and the protein blots were visualized with the enhanced chemiluminescent detection system (GE, Fairfield, CT, USA). Protein bands were visualized using ImageJ 1.45S software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data analysis in the present study was performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Results were presented as the mean ± standard deviation. The associations between SNHG20 expression and clinicopathological factors were analyzed using the χ^2 test. The significance of differences between groups was analyzed with the Student's t-test or one-way ANOVA followed by a Student-Newman-Keuls-q test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Increased SNHG20 expression is determined in OSCC tissues and associates with histological differentiation and TNM stage. The expression of SNHG20 in 40 human OSCC tissue specimens and matched adjacent non-tumor tissue specimens was examined. As depicted in Fig. 1A, the SNHG20 expression was significantly increased in OSCC tissue specimens, compared with in the matched adjacent non-tumor tissue

Table I. The association between SNHG20 expression and clinicopathological characteristics in patients with oral squamous cell carcinoma.

Clinicopathological characteristics	Total	SNHG20 expression		P-value
		Reduced (n=20)	Increased (n=20)	
Age				0.525
≤60	22	12	10	
>60	18	8	10	
Sex				0.519
Female	16	7	9	
Male	24	13	11	
Smoking status				0.527
No	20	11	9	
Yes	20	9	11	
Tumor site				0.744
Tongue	15	8	7	
Non-tongue	25	12	13	
T stage				0.197
T1-T2	20	14	6	
T3-T4	20	6	14	
Differentiation (13)				0.025[a]
Well and moderately	23	15	8	
Poorly	17	5	12	
Lymph node metastasis				0.185
No	26	15	11	
Yes	14	5	9	
TNM stage (13)				0.011[a]
I-II	22	15	7	
III-IV	18	5	13	

[a]P<0.05. SNHG20, small nucleolar RNA host gene 20; TNM, Tumor-Node-Metastasis.

specimens (P<0.05). Furthermore, SNHG20 expression was significantly increased in three OSCC cell lines (SCC4, HN4 and SCC15), compared with in the NHOK cell line (P<0.05; Fig. 1B). In order to understand the clinical significance of SNHG20 expression in patients, the association between SNHG20 expression and clinicopathological factors were analyzed with the χ^2 test. The results demonstrated that SNHG20 overexpression was significantly associated with a reduced histological differentiation and advanced TNM stage (III-IV stage; P<0.05; Table I) (13).

Increased SNHG20 expression is a predictor of overall survival (OS) of patients of OSCC. Survival plots were performed with the Kaplan-Meier method and compared with log-rank tests. The results demonstrated that increased SNHG20 expression in patients with OSCC reduced their OS, compared with in patients with reduced SNHG20 expression (Fig. 1C). In univariate analysis, the results indicated that poor tumor differentiation, advanced clinical stage and increased SNHG20 expression were independent predictors of the OS of patients with OSCC, and these associations were significant (P<0.05; Table II). Furthermore, multivariate Cox proportional hazards regression analysis demonstrated that poor tumor differentiation, advanced clinical stage and increased SNHG20 expression were independent predictors of the OS of patients with OSCC, and these associations were significant (P<0.05; Table II); thus, these results indicated that increased SNHG20 expression may serve as an independent predictor for the OS of patients with OSCC.

Decreased expression of SNHG20 expression inhibits cell proliferation. The impact of SNHG20 expression on OSCC cell proliferation was also examined. SNHG20 expression was knocked down in two increased SNHG20 expression OSCC cell lines (SCC4 and SCC15 cells). The knockdown efficiency of two siRNAs was increased and depicted in Fig. 1D. The cell proliferation ability was detected using CCK-8 cell proliferation and cell colony formation assays. The CCK-8 results indicated that decreased expression of SNHG20 significantly inhibited the cell proliferation capacity of SCC4 and SCC15 cells, compared with their respective negative control groups (P<0.05; Fig. 2A and B). Additionally, the colony forming assay results demonstrated

Table II. Univariate and multivariate Cox proportional hazards analysis of SNHG20 expression and OS in patients with oral squamous cell carcinoma.

	Univariate Cox analysis		Multivariate Cox analysis	
Clinicopathological characteristics	RR (95% CI)	P-value	RR (95% CI)	P-value
Age	0.788 (0.355-1.477)	0.655		
Sex	1.088 (0.546-1.877)	0.565		
Smoking status	0.927 (0.443-1.788)	0.588		
Tumor site	1.156 (0.577-1.993)	0.422		
Non-tongue	0.655 (0.301-1.444)	0.723		
T stage	0.756 (0.404-1.466)	0.688		
Differentiation (13)	2.131 (1.587-2.977)	0.001[a]	1.896 (1.448-2.745)	0.002[a]
Lymph node metastasis	1.223 (0.779-1.987)	0.332		
TNM stage (13)	2.289 (1.642-3.224)	0.001[a]	1.966 (1.246-3.009)	0.001[a]
SNHG20	2.556 (1.822-3.663)	0.001[a]	2.077 (1.388-3.244)	0.001[a]

[a]P<0.05. TNM, Tumor-Node-Metastasis; SNHG20; small nucleolar RNA host gene 20; CI, confidence interval; RR, relative risk.

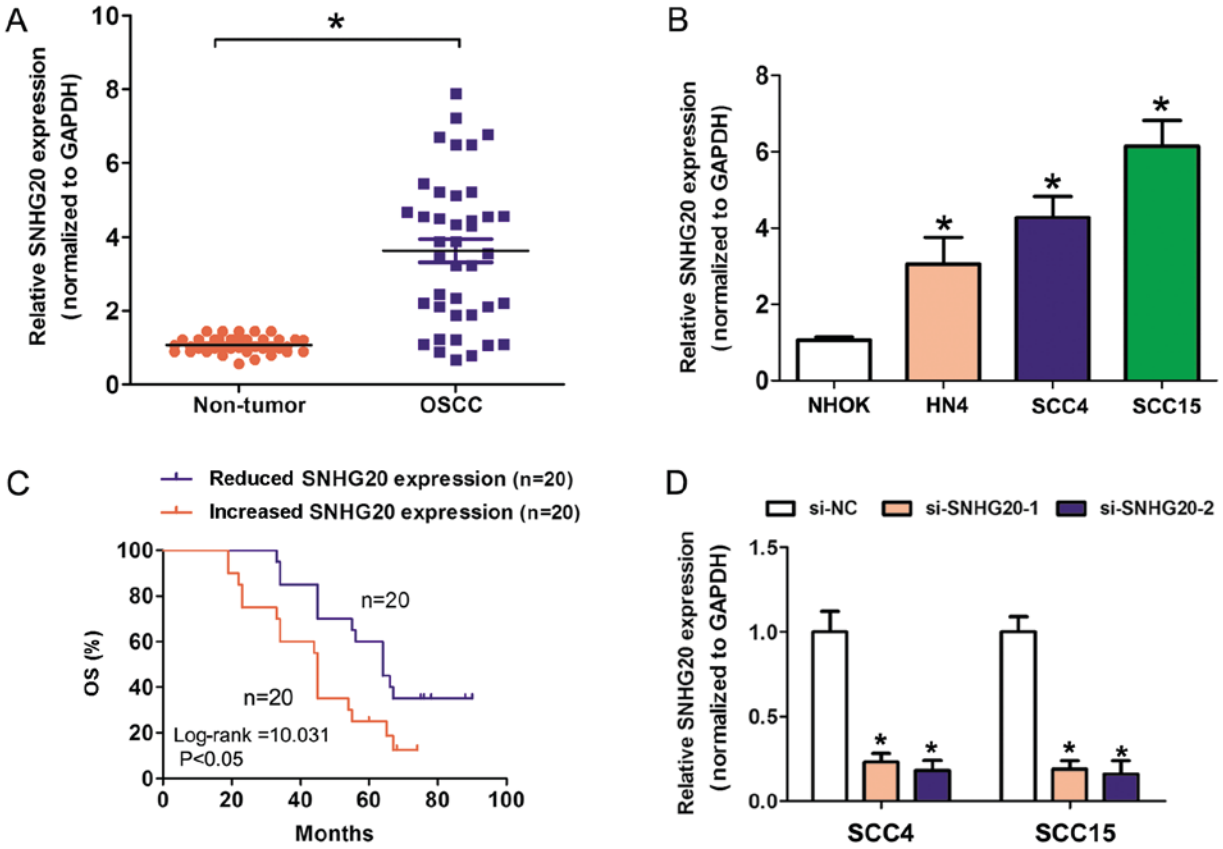

Figure 1. Increased SNHG20 expression was determined in OSCC tissues and is associated with a poor OS. (A) SNHG20 expression was significantly increased in OSCC tissues, compared with in adjacent non-tumor tissues with a RT-qPCR assay. (B) SNHG20 expression was significantly increased in three human OSCC cell lines (SCC4, HN4 and SCC15), compared with in the NHOK cell line. (C) Kaplan-Meier analysis for the effects of SNHG20 expression on the OS of patients. (D) The relative expression of SNHG20 was detected with a RT-qPCR assay when SCC4 and SCC15 cells were transfected with si-NC, si-SNHG20-1 or si-SNHG20-2. Error bars represent mean ± standard deviation for ≥3 independent experiments, *P<0.05 compared with si-NC. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OSCC, oral squamous cell carcinoma; SNHG20, small nucleolar RNA host gene 20; NHOK, normal human oral keratinocyte; si-NC, small interfering-negative control; OS, overall survival.

that decreased expression of SNHG20 significantly reduced the number of SCC4 and SCC15 cell colonies, compared with their respective negative control groups (P<0.05; Fig. 2C and D). It was also indicated that knockdown of SNHG20 downregulated the expression of PCNA and Ki67 in SCC4 and SCC15 cells, compared with their respective negative control groups

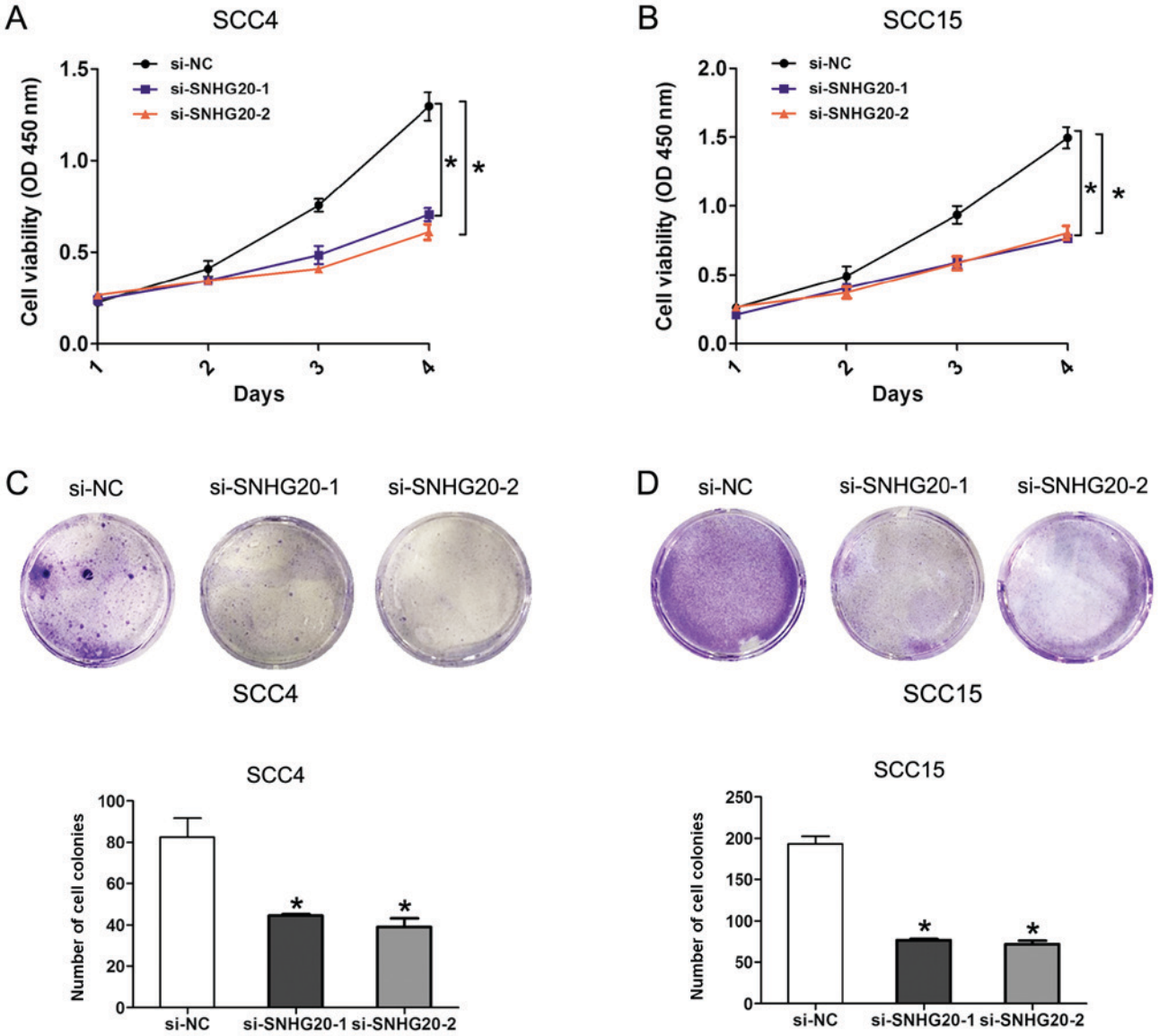

Figure 2. Decreased expression of SNHG20 expression inhibits cell proliferation. Cell Counting kit-8 cell proliferation assays were applied to examine the cell proliferation ability when (A) SCC4 and (B) SCC15 cells were transfected with si-NC, si-SNHG20-1 or si-SNHG20-2. Cell colony formation assays were applied to examine the cell proliferation ability when (C) SCC4 and (D) SCC15 cells were transfected with si-NC, si-SNHG20-1 or si-SNHG20-2. Error bars represent mean ± standard deviation from ≥3 independent experiments, *P<0.05 compared with si-NC. SNHG20, small nucleolar RNA host gene 20; si-NC, small interfering-negative control; OD, optical density.

(Fig. 3A-D). The present results demonstrated that decreased expression of SNHG20 inhibited cell proliferation.

Discussion

Recent evidence demonstrated that lncRNAs act as regulatory molecules that mediate cellular processes, including chromatin remodeling, transcription, post-transcriptional modifications and signal transduction (15,16). lncRNAs could affect cell proliferation, cell apoptosis, differentiation and invasion, which serves crucial roles in biological processes (17,18). lncRNAs in OSCC progression could provide diagnostic and therapeutic strategies for this disease. SNHG20, a recently determined RNA molecule, is identified as an oncogene in a number of tumor types including colorectal cancer and gastric cancer. However, to the best of our knowledge, the prognostic value and functional role of SHNG20 in OSCC remains unknown. In the present study, the results indicated that SNHG20 expression was increased in OSCC tissue specimens, compared with in adjacent non-tumor tissue specimens. SNHG20 overexpression was associated with reduced histological differentiation and advanced clinical stage. Multivariate Cox proportional hazards regression analysis demonstrated that increased SNHG20 expression was an independent predictor for the OS of patients with OSCC.

In the previous study, SNHG20 was determined to be increased in colorectal cancer tissues, compared with in corresponding non-tumor tissues, and high expression of SNHG20 was notably associated with advanced TNM stage in patients with colorectal cancer (11). Additionally, upregulation of SNHG20 predicts a poor overall survival rate in patients with hepatocellular carcinoma. Knockdown of SNHG20 in SK-Hep-1 cells significantly inhibited cellular proliferation, migration and invasion (19). SNHG20 promotes cell proliferation, invasion and migration of breast cancer cells via

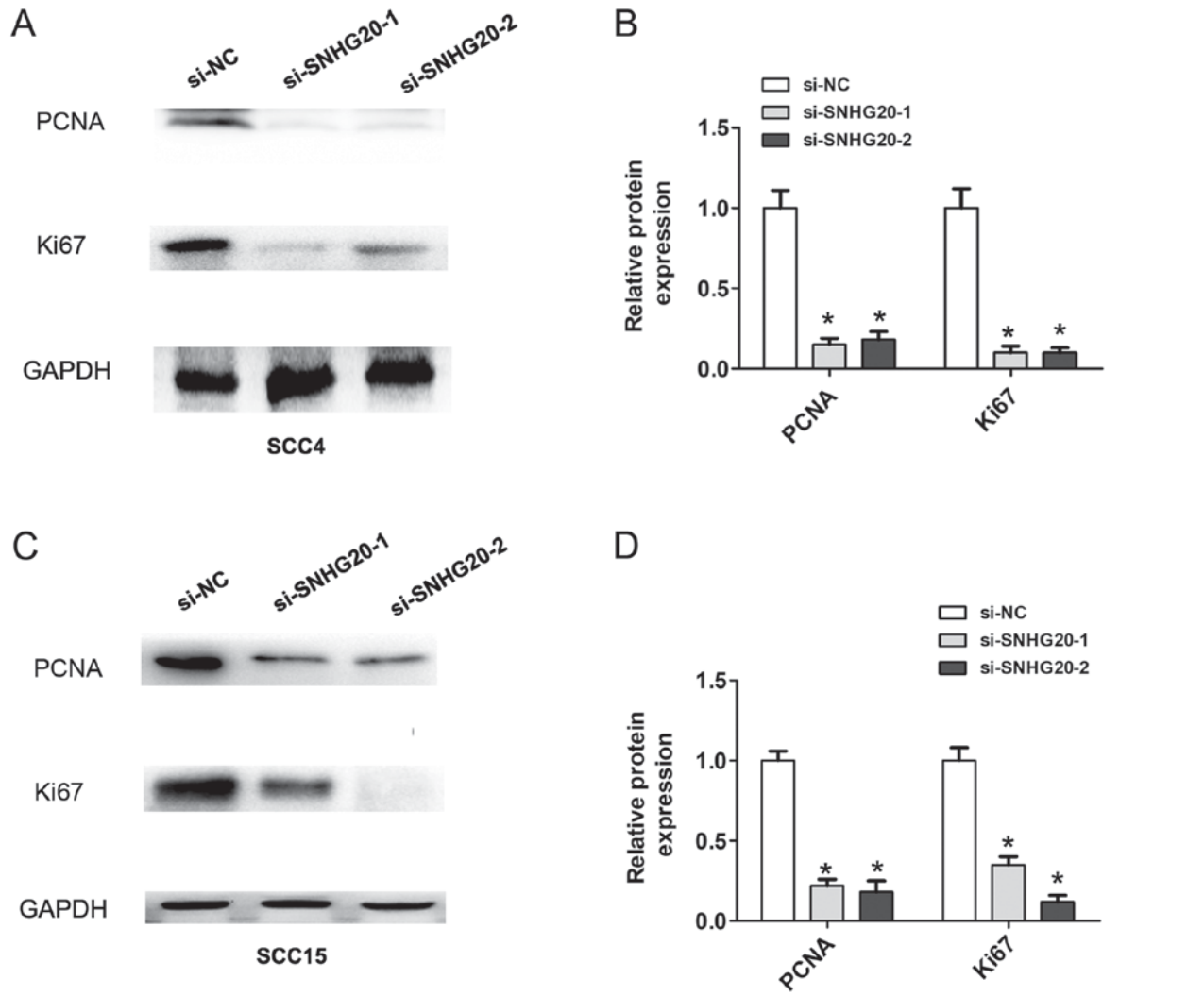

Figure 3. Decreased expression of SNHG20 expression inhibits cell proliferation-associated proteins PCNA and Ki67 expressions. (A) The expression of PCNA and Ki67 was detected using western blot analysis when SCC4 cells were transfected with si-NC, si-SNHG20-1 and si-SNHG20-2. (B) Protein quantitative analysis of SCC4 cells when transfected with si-NC, si-SNHG20-1 and si-SNHG20-2. *P<0.05 compared with si-NC. (C) The expression of PCNA and Ki67 was detected using western blot analysis when SCC15 cells were transfected with si-NC, si-SNHG20-1 or si-SNHG20-2. (D) Protein quantitative analysis of SCC15 cells transfected with si-NC, si-SNHG20-1 and si-SNHG20-2. *P<0.05 compared with si-NC. PCNA, proliferating cell nuclear antigen; SNHG20, small nucleolar RNA host gene 20; si-NC, small interfering-negative control.

regulating miR-495 (20). Consistently, the present results demonstrated that decreased expression of SNHG20 expression inhibited cellular proliferation capacity and cell colony formation ability. Additionally, it was indicated that decreased expression of SNHG20 downregulated the expression of PCNA and Ki67 in OSCC cells. PCNA and Ki67 are cell proliferation markers, whose rate of synthesis is directly associated with the rates of cellular proliferation and DNA synthesis (21). The present results indicated that SNHG20 may affect the PCNA and Ki67 expression by potential mechanisms. These results demonstrated that decreased SNHG20 expression inhibited cell proliferation. Furthermore, in the future *in vivo* experiments should be performed, in order to demonstrate the role of SNHG20 in OSCC.

In conclusion, in the present study, it was demonstrated that SNHG20 expression was elevated in OSCC tissues and cells. Increased SNHG20 expression functions as a prognostic predictor for OSCC tissues. Furthermore, it was demonstrated that decreased SNHG20 expression inhibited cell proliferation of OSCC; thus, these results indicated that SNHG20 may act as a prognostic predictor for OSCC and a target of OSCC treatment. Additionally, the precise molecular mechanisms underlying OSCC progression require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

PG contributed to the design of the study and was responsible for project design, organization, manuscript writing, the supervision of experimental progress and final approval of the version to be published. PG, RF and TG conducted the molecular experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol of the present study was approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine, and written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all patients and consent for publication.

Competing interests

The authors declare that they have no competing interests.

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