Downregulation of nucleolar and spindle-associated protein 1 expression suppresses liver cancer cell function

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Abstract. The aim of the present study was to determine the role of nucleolar and spindle-associated protein 1 (NuSAP1) in human liver cancer. NuSAP1 expression was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and immunohistochemistry in hepatocellular carcinoma (HCC) and adjacent tissues. The expression of NuSAP1 gene was detected by RT-qPCR in liver cancer cell lines. Expression information for NuSAP1 was determined using the UALCAN and Oncomine databases. The Kaplan-Meier plotter and The Cancer Genome Atlas databases were used to obtain overall survival data for liver cancer. Liver cancer cell lines HepG2 and Huh-7 were transfected with lentiviral particles to silence the endogenous NuSAP1 gene expression. RT-qPCR and western blotting were performed to confirm the silencing efficiency. Cell Counting Kit-8 was used to estimate the effects of NuSAP1 silencing on HepG2 and Huh-7 cell proliferation. Cell cycle and apoptosis analyses were performed using flow cytometry. Cell invasion was assessed using the Transwell assay with microscopy imaging. The results revealed that the NuSAP1 expression levels in HCC tissues were significantly increased compared with the adjacent tissues. The survival time of patients with HCC with a high NuSAP1 expression was markedly decreased compared with that of patients with HCC with a low expression level of NuSAP1. Functional studies revealed that NuSAP1 silencing significantly reduced HepG2 and Huh-7 cell proliferation and invasion. Increased apoptosis and cell cycle arrest at the G1 phase were observed following NuSAP1 knockdown. NuSAP1 silencing significantly inhibited the mRNA expression of DNA methyltransferase but not glioma-associated oncogene. NuSAP1 contributed to liver cancer development by reducing apoptosis and promoting cell cycle progression. The abnormal expression level of NuSAP1 may serve a role in promoting liver cancer progression.

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Introduction

Hepatocellular carcinoma (HCC), which accounts for ~80% of adult primary liver cancer, is the most lethal form of liver cancer worldwide (1), with the prevalence and incidence rates rising worldwide annually (2). Early diagnosis of liver cancer is challenging due to the limited amount of clinical studies and accurate biomarkers (3). In addition, the lack of specific symptoms in the early stages further results in poor prognosis (3).

Nucleolar and spindle-associated protein 1 (NuSAP1) is an important microtubule and chromatin-binding protein. It has been demonstrated to serve a role in microtubule crosslink during mitosis (4-6), regulate the function of kinetochore microtubules (7), and dominate chromosome oscillation (8). Furthermore, NuSAP1 regulates spindle assembly, chromosome segregation and cytokinesis (4,9). Previous studies have reported that NuSAP1 is involved in malignancies, including melanoma (10,11), breast cancer (12,13), glioblastoma (14), HCC (15), prostate cancer (16), glioblastoma multiforme (17), astrocytoma (18) and meningioma (19). Cui *et al* (20) revealed that NuSAP1 may act as one of the core modules in the suppression of HCC by silymarin. In addition, it has been reported that, during cell division, NuSAP1 acted as a co-factor for the E3 SUMO-protein ligase RanBP2-Ran GTPase-activating protein 1-SUMO-conjugating enzyme UBC9 complex and participated in accurate chromosome segregation (21). The above studies indicated that NuSAP1 serves a role in cancer development. To further understand the role of NuSAP1 in

liver cancer, the current study investigated the effects of the downregulation of NuSAP1 expression in liver cancer cell lines. Functional studies confirmed the biological significance of the NuSAP1 gene in liver cancer.

Materials and methods

Patient and tissue samples. Tumorous and adjacent non-tumorous human liver specimens were collected from 47 patients who underwent surgery for HCC at the Nantong Third People's Hospital Affiliated to Nantong University (Nantong, China) between September 2015 and July 2016. All patients in this study were diagnosed with primary HCC. No preoperative radiotherapy or chemotherapy was administered to these patients. Histopathological diagnosis of all patients with HCC was completed by the Department of Pathology. The pathological stage of HCC was determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) Classification (22). The baseline characteristics of the participants were summarized in a previous study (23). All tissues used in the current study were frozen immediately following dissection and stored in liquid nitrogen.

Databases. The expression of NuSAP1 in patients with HCC was analyzed using the multiple cancer microarray datasets available from Oncomine (www.oncomine.org) (24). The Cancer Genome Atlas expression information for NuSAP1 was obtained using UALCAN (www.ualcan.path.uab.edu) (25). A 120-month follow-up study was performed, using HCC patient information available from the Kaplan-Meier plotter database (www.kmplot.com) (26), to verify the association between NuSAP1 expression and prognosis in patients with HCC. Furthermore, a 80-month follow-up study was also performed using OncoLnc (www.oncolnc.org) (27).

Cell lines. LO2, HepG2, Huh-7 and SNU-423 cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ScienCell Research Laboratories, Inc., San Diego, CA, USA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Inc.) and maintained at 37˚C with 50 ml/l CO_2.

Lentiviral infection. One day prior to infection, the HepG2 or Huh-7 cell suspensions were seeded onto six-well plates, at a density of $1x10^5$ cells/well. Two groups included, the siNuSAP1 group, transfected with NuSAP1-siRNA green fluorescent protein (GFP) lentivirus, and the control group, transfected with a scrambled siRNA GFP lentivirus. The following day, DMEM with 10% v/v FBS (complete medium) was removed and replaced with Enhanced Infection Solution (1 ml/well; Shanghai GeneChem Co., Ltd., Shanghai, China), and polybrene (Shanghai GeneChem Co., Ltd.) was diluted to a final concentration of 5 μg/ml with Enhanced Infection Solution. For each cell line, different multiplicity of infection (MOI) values (20, 40, 60, 80, 100 and 120) were tested, and the final MOI value used in the present study was determined when the efficiency of infection reached 80% 3 days post-infection. HepG2 cells were infected at a MOI of 120. Huh-7 cells were infected at a MOI of 60. After swirling the plate gently to mix the cells, the plate was placed in an incubator with 50 ml/l CO_2 at 37˚C. After 8 h, the medium was removed and replaced by the complete medium. Three days post-transfection, GFP expression was observed in five randomly-selected fields using a fluorescence microscope (magnification, x200). Cells with transfection efficiency >80% on day 3 were selected for subsequent analysis. Lentiviruses were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The following target siRNA sequences were used: Scrambled control, 5'-TTCTCCGAACGTGTCACGT-3', siNuSAP4-1, 5'-ATAAGCGTTCACTGACCAA-3', and siNuSAP1 6-1, 5'-CCTTAAAGCTCAAATTCTT-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) from HCC tumor tissues, adjacent tissues and liver cancer cell lines. The RNA purity and concentration were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The Prime Script™ RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used for the RT of RNA, according to the manufacturer's protocol. The reactions were performed using a Bio-Rad Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). SYBR® Green Master Mix (Vazyme, Piscataway, NJ, USA) was used for the qPCR. The following primers were used: NuSAP1 forward, 5'-TCA TTTCCTTTTCTTGCCTCA-3' and reverse, 5'-CCCTCA AGTACAGTGACCTGC-3'; glioma-associated oncogene (GLI1) forward, 5'-AACCCTTGGAAGGTGATATGTC-3' and reverse, 5'-TTCATACACAGATTCAGGCTCA-3'; DNA methyltransferase (DNMT1) forward, 5'-CGGCAGACCATC AGGCATTCTAC-3' and reverse, 5'-CACACCTCACAG ACGCCACATC-3'; β-actin forward, 5'-GGACTTCGAGCA AGAGATGG-3' and reverse, 5'-AGGAAGGAAGGCTGG AAGA-3'. The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; 40 cycles of 10 sec at 95˚C and 30 sec at 60˚C. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (28).

Western blotting. Tissues and cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China). The supernatants of centrifuged lysates (14,000 x g for 5 min at 4˚C) were diluted in 5X Laemmli SDS sample buffer (Beyotime Institute of Biotechnology) at a 4:1 ratio. Total protein was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology) and then boiled at 95˚C for 5 min. Proteins (35 μg/lane) were separated by SDS-PAGE on 10% gels. The separated proteins were transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA), and incubated with primary antibodies against NuSAP1 (cat. no. 12024-I-AP; 1:2,000) and β-actin (cat. no. HRP-60008; 1:2,000; both ProteinTech Group, Inc., Chicago, IL, USA) overnight at 4˚C. Following washing with tris-buffered saline and Polysorbate 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. A00098; 1:2,000; GenScript Co., Ltd., Nanjing, China) for 2 h at room temperature. The protein-antibody complexes were visualized using the SuperSignal

West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific, Inc.) and exposed to a medical X-ray film (Denville Scientific Inc., Holliston, MA, USA). Proteins were quantified using the Multi Gauge software (version 3.0; Fujifilm Dimatix Inc, Santa Clara, CA, USA).

Immunohistochemistry (IHC). Tissues were fixed in 10% formalin for 12 h at 4˚C and embedded in paraffin. Paraffin-embedded tissue samples (6-μm-thick) were cut. All sections were heated in roaster at 65˚C for 1 h, and then deparaffinized in dimethylbenzene and rehydrated in a descending alcohol series at room temperature. Antigen retrieval was performed by boiling the tissue sections in citrate buffer (pH 6.0) in a microwave for 30 min. Endogenous peroxidase activity was quenched by treatment with methanol and 3% hydrogen peroxide for 15 min at room temperature and background staining was blocked for 1 h at room temperature with 10% non-immune goat serum (Thermo Fisher Scientific, Inc.). Tissue sections were incubated with primary antibody against NuSAP1 (cat. no. 12024-I-AP; 1:200; ProteinTech Group, Inc.) overnight at 4˚C. Following washing with PBS, the tissue sections were incubated with a horseradish peroxidase-conjugated secondary antibody (Shanghai Long Island Biotec Co., Ltd, Shanghai, China) for 30 min at room temperature. Tissue sections were subsequently washed with PBS and stained with 3,3'-diaminobenzidine (DAB; Maixin-Bio, Guangzhou, China) for 60 sec. Tissue sections were counterstained with Hematoxylin for 15 sec at room temperature for nuclear staining. Sections without the primary antibody served as negative controls. Histological sections of fixed tissue samples were observed using a light microscope (magnification, x200; Leica Microsystems GmbH, Wetzlar, Germany).

Cell proliferation assay. A total of 100 μl of cell suspension (3,000 cells/well) were seeded into each well of a 96-well plate. The plate was subsequently pre-incubated for 12 h in a humidified incubator at 37˚C with 5% CO_2. This time point was defined as day 1. Subsequently, the plate was incubated for 0, 24, 48 or 72 h in the incubator, followed by the addition of 10 μl of Cell Counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) to each well of the plate, and further incubation for 1.5 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Cell cycle analysis. One day prior to transfection, HepG2 or Huh-7 cells were seeded into six-well plates. Three days post-transfection, cells were washed with PBS, collected and fixed in 70% ice-cold ethanol and directly stored in 70% ethanol overnight at -20˚C. Prior to analysis, the cells were incubated in 500 μl sample buffer [50 μg/ml propidium iodide (Merck KGaA, Darmstadt, Germany) and 0.25 mg/ml of RNase A (Takara Bio, Inc., Otsu, Japan)] for 30 min at room temperature. The proportion of cells in each phase was detected using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Apoptosis analysis. One day prior to the transfection, HepG2 or Huh-7 cells were seeded into six-well plates. Three days post-transfection, cells were harvested and washed with PBS. Cell apoptosis was assessed using the Annexin V-Allophycocyanin Apoptosis Detection kit I (BD Biosciences). Cells were centrifuged (200 x g for 5 min at room temperature) and resuspended in 500 μl of binding buffer to the density of $1x10^6$ cells/ml. Subsequently, 100-μl cell suspension was incubated with 5 μl 7-aminoactinomycin D and 5 μl Annexin V-fluorescein isothiocyanate for 15 min at room temperature, in the dark. Apoptotic cells were detected using a BD FACSCalibur Flow cytometry (BD Biosciences) and analyzed using FlowJo software.

Transwell invasion assay. Transwell filters were coated with Matrigel (3.9 μg/μl; 60-80 μl) on the upper surface of the polycarbonic membrane (diameter, 6.5 mm; pore size, 8 μm). The Matrigel solidified following incubation at 37˚C for 6 h. Cells ($1x10^5$) suspended in 200 μl serum-free DMEM were added into the upper compartment of the chamber. Simultaneously, 600 μl complete medium was added into the bottom compartment of the chamber. The medium was removed from the upper chamber following incubation for 48 h at 37˚C with 5% CO_2. The Matrigel was cleaned with a cotton swab. The cells migrated from the Matrigel into the pores of the filter were fixed with 100% methanol, stained with crystal violet (Beyotime Institute of Biotechnology) for 30 min at room temperature, washed with PBS and dried at room temperature. The cells invading through the Matrigel were observed using an inverted microscope (magnification, x200). Each assay was repeated three times.

Statistical analysis. GraphPad Prism was used for data analysis (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test and two-way analysis of variance followed by Tukey's post hoc test were used in the current study. Significance of NuSAP1 expression in association with clinicopathological parameters was calculated using the Chi-square test. A paired t-test was used to analyze the differences of NuSAP1 expression levels between HCC tissues and corresponding adjacent normal tissues. $P<0.05$ was considered to indicate a statistically significant difference. All data are presented as the mean ± standard error of the mean. All the experiments were repeated at least three times.

Results

NuSAP1 is overexpressed in HCC tissues and HepG2 and Huh-7 cell lines. The expression of NuSAP1 in HCC was analyzed using the multiple cancer microarray datasets available from Oncomine and it was determined that the mRNA expression levels of NuSAP1 in HCC tissues were markedly increased compared with those in the normal tissues (Fig. 1A). The Cancer Genome Atlas expression information for NuSAP1 was obtained using UALCAN (Fig. 1B). To improve the understanding of the clinical relevance of NuSAP1 expression in HCC, the association between NuSAP1 expression and clinicopathological parameters was examined (Table I). According to the median value of relative NuSAP1 mRNA expression in HCC tissues, 47 HCC patients were subsequently divided into NuSAP1-high (n=24) and NuSAP1-low groups (n=23). The statistical analysis revealed that a high level of NuSAP1

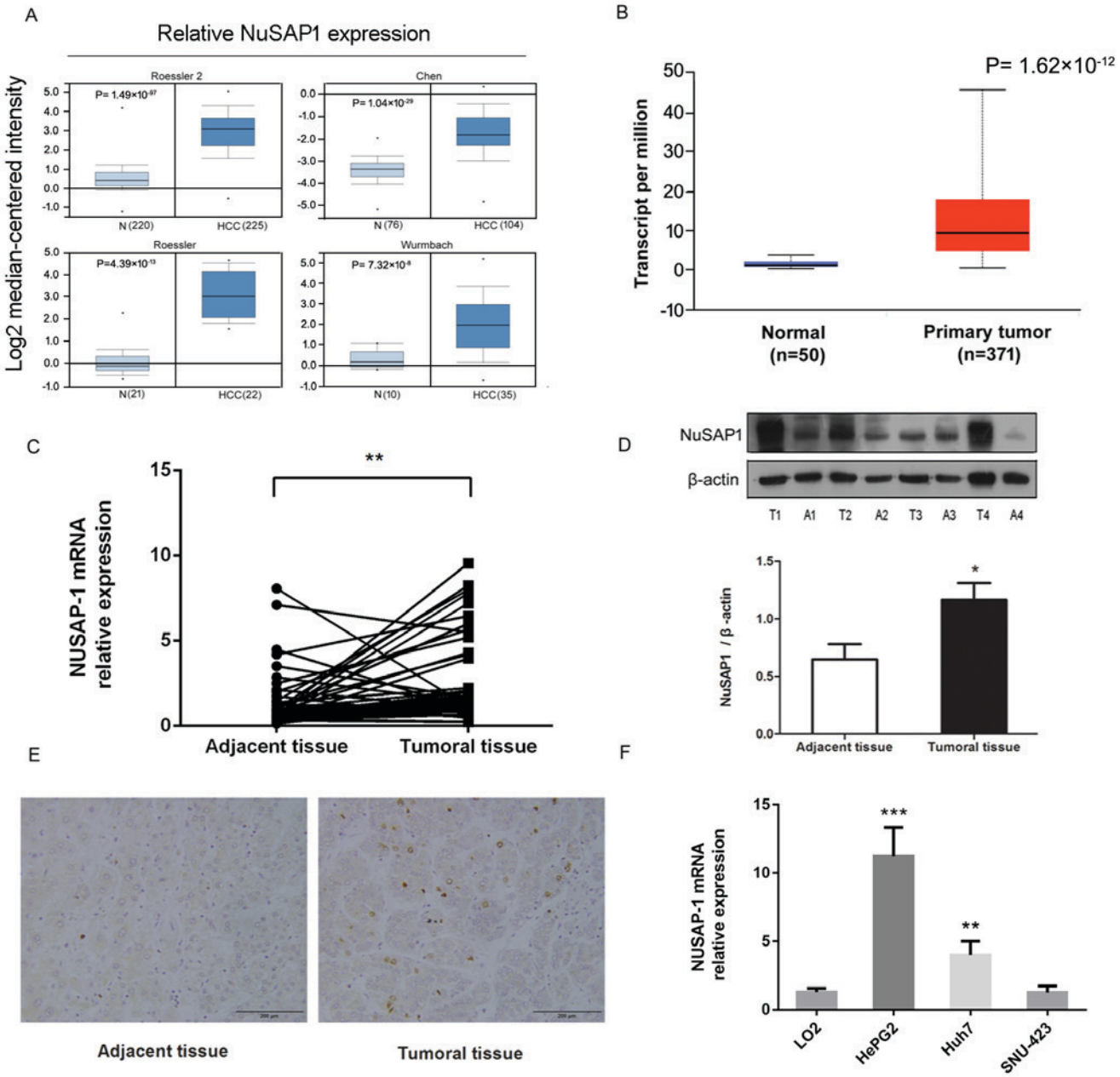

Figure 1. NuSAP1 is overexpressed in HCC tissues and HepG2 and Huh-7 cell lines. (A) NuSAP1 is overexpressed in human HCC tissues, as compared with that in normal tissues, in multiple cancer microarray datasets available from Oncomine. (B) NuSAP1 is overexpressed in human HCC tissues compared with normal tissues, in datasets available from The Cancer Genome Atlas. (C) mRNA expression levels of NuSAP1 in HCC (n=47) and paired adjacent tissue samples (n=47) were determined by reverse transcription-quantitative polymerase chain reaction. (D) Protein expression levels of NuSAP1 in HCC (n=47) and paired adjacent tissue samples (n=47) were determined by western blotting. Gray values of the western blot were quantified. Data were normalized to β-actin. (E) Expression level of NuSAP1 in HCC and paired adjacent tissues determined by immunohistochemistry. Scale bar, 200 μm. (F) Relative NuSAP1 mRNA levels in LO2, HepG2, Huh-7 and SNU-423 cell lines were determined by reverse transcription-quantitative polymerase chain reaction. β-actin was used as the internal control. *P<0.05 vs. with adjacent tissue. **P<0.01 and ***P<0.001 vs. LO2 cell line. HCC, hepatocellular carcinoma; NuSAP1, nucleolar and spindle-associated protein 1; T, tumor tissue; A, adjacent tissue.

expression in HCC was significantly associated with the TNM stage (P=0.011) and lymph node metastasis (P=0.019). However, NuSAP1 expression was not associated with the other clinicopathological features, including age (P=0.77), sex (P=0.371), serum level of α-fetoprotein (P=0.494), hepatitis B virus infection (P=0.286) and tumor size (P=0.248). The current data indicate that elevated NuSAP1 expression may serve a role in HCC progression, and may be a valuable biomarker for this disease. To investigate whether the NuSAP1 expression was altered in HCC tissues, RT-qPCR and western blotting were used to detect the NuSAP1 levels in 47 paired tumor and adjacent tissues. The mRNA and protein levels ofNuSAP1 in the tumor tissues were significantly increased compared with the adjacent tissues (Fig. 1C and D). Furthermore, the expression level of NuSAP1 was detected by IHC in 47 paired tumor and adjacent tissues (Fig. 1E). The results revealed that the expression of NuSAP1 was increased in HCC tissues compared with adjacent tissues. To reveal the potential role of NuSAP1 in HCC, NuSAP1 expression was examined in four liver cancer cell lines: HepG2, Huh-7 and

Table I. Association between NuSAP1 expression and the clinicopathological characteristics of the 47 patients with HCC.

Characteristics	Total, n	NuSAP1 expression		P-value
		Low, n=23	High, n=24	
Age, years				
≤56	28	13	15	0.77
>56	19	10	9	
Gender				
Female	18	7	11	0.371
Male	29	16	13	
Serum AFP, ng/ml				
≤400	36	19	17	0.494
>400	11	4	7	
HBV infection				
Positive	38	17	21	0.286
Negative	9	6	3	
TNM stage, I/II/III				
I+II	32	20	12	0.011
III	15	3	12	
Tumor size, cm				
≤5	24	14	10	0.248
>5	23	9	14	
Lymph node metastasis				
Positive	20	14	6	0.019
Negative	27	9	18	

HCC, hepatocellular carcinoma; AFP, α-fetoprotein; HBV, hepatitis B virus; TNM, tumor, node, metastasis.

SNU-423 as well as the normal human hepatic cell line LO2 (Fig. 1F). The expression of NuSAP1 was significantly higher in HepG2 and Huh-7 cells compared with LO2 cells. There were no significant changes in NuSAP1 expression level in the SNU-423 cell line compared with LO2. Therefore, the HepG2 and Huh-7 cell lines were selected for future investigation.

High NuSAP1 expression is associated with poor prognosis in HCC. To verify the association between NuSAP1 expression and prognosis in HCC, a 120-month follow-up study was performed using HCC patient information available from the Kaplan-Meier plotter database (Fig. 2A) and the result was consistent with OncoLnc (Fig. 2B). The association between the NuSAP1 expression level and prognosis was analyzed. Patients with a higher NuSAP1 expression level exhibited a markedly shorter overall survival compared with those with a lower NuSAP1 expression level.

NuSAP1 silencing inhibits the proliferation and invasion of HepG2 and Huh7 cells. Following siRNA lentiviral transfection, RT-qPCR was performed to determine the expression levels of NuSAP1 in different groups. The results revealed that NuSAP1-siRNA (siRNA 4-1 and siRNA 6-1) decreased the expression of endogenous NuSAP1 mRNA in HepG2 cells (Fig. 3A). Simultaneously, the protein expression of NuSAP1 in NuSAP1-siRNA-treated cells was suppressed compared with cells transfected with the scrambled control (Fig. 3B and C). The results revealed that siRNA 6-1 exhibited a higher efficiency compared with siRNA 4-1. Therefore, siRNA 6-1 was selected for further investigation. In order to determine whether NuSAP1 exerted a tumor facilitating function, the expression of NuSAP1 was suppressed through a lentiviral vector system using siRNA 6-1 in HepG2 and Huh-7 cells with high endogenous NuSAP1 levels. The NuSAP1 expression levels were determined by RT-qPCR and western blotting. To investigate whether NuSAP1 served a role in cell growth in HepG2 and Huh-7 cells, cell proliferation was detected using the CCK-8, and invasive potential was determined by the Transwell assay. The results revealed that the downregulation of NuSAP1 markedly suppressed cell proliferation (Fig. 4A and B) and the invasive capability (Fig. 4C and D) of both HepG2 and Huh-7 cells.

NuSAP1 silencing induces cell cycle progression and cell apoptosis. To investigate the growth-suppressing effect of NuSAP1-siRNA on HepG2 and Huh-7 cells, the cell cycle distribution was analyzed by flow cytometry. NuSAP1-siRNA significantly increased the fraction of G1-phase cells in both HepG2 and Huh-7 cells compared with the control group, and decreased the proportion of S-phase cells in the NuSAP1-siRNA group in Huh7 cells (Fig. 5A and B). The results indicated that NuSAP1 silencing may induce cell cycle

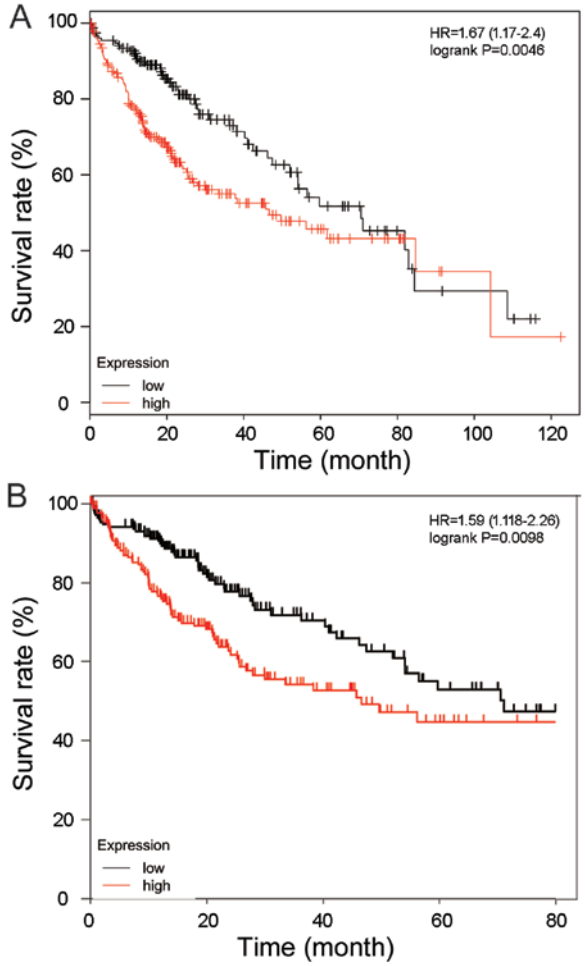

Figure 2. Association between the expression level of nucleolar and spindle-associated protein 1 and prognosis of patients with hepatocellular carcinoma. (A) The difference in survival between the high-and low-level groups was analyzed by the log-rank test from the Kaplan-Meier plotter database (P=0.0046). (B) The difference in survival between the high-and low-level groups was analyzed by the log-rank test from The Cancer Genome Atlas database (P=0.0098).

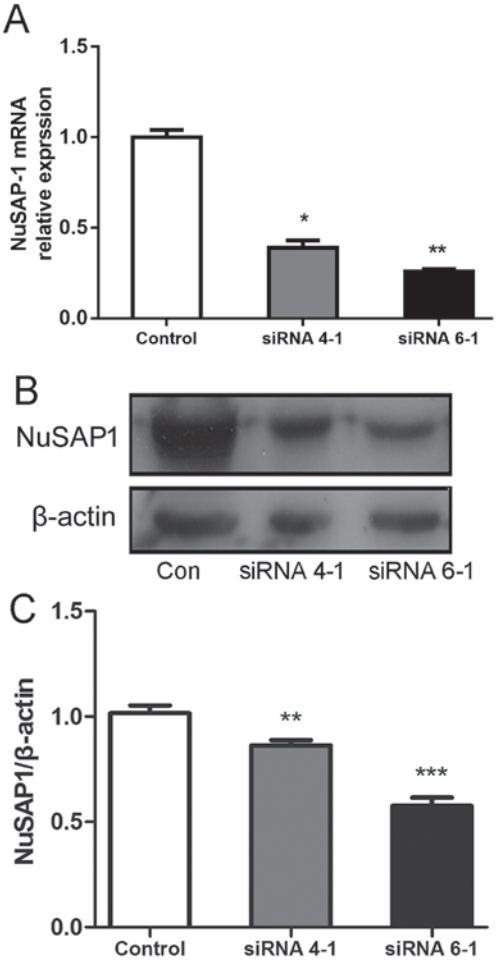

Figure 3. Downregulation of NuSAP1 by lentiviral infection. (A) A total of 72 h after lentiviral transfection (siRNA 4-1 and siRNA 6-1), the relative NuSAP1 mRNA expression was significantly inhibited in HepG2 NuSAP1-siRNA-silenced cells compared with HepG2 negative control cells, as determined by reverse transcription-quantitative polymerase chain reaction. (B) Western blotting of NuSAP1-depletion efficiency in HepG2 cells. (C) Gray value analysis of western blotting. Data were normalized to β-actin, which was used as the internal control. *P<0.05, **P<0.01 and ***P<0.001 vs. the control. NuSAP1, nucleolar and spindle-associated protein 1; siRNA, small interfering RNA.

arrest at the G1 phase. The current study further investigated whether NuSAP1 silencing was associated with apoptosis in HepG2 and Huh-7 cells. The number of cells with NuSAP1 gene silencing that exhibited apoptosis was significantly higher compared with the control group (Fig. 5C and D). Lentiviral transfection exhibited considerable infection efficiency toward cells, however it has intrinsic cell toxicity (data not shown). The MOI value used in the current experiment resulted in the most efficient knockdown ratio of NuSAP1 at both mRNA and protein levels (data not shown). Equal MOI was used for the scrambled control group and, therefore, the conclusion was not affected. The above data suggested that the silencing of NuSAP1 interrupted cell cycle progression and affected cell survival.

NuSAP1 silencing inhibits the DNMT1 but not the GLI 1 gene expression in liver cancer cells. A prioritized set of 841 enriched GLI1-binding regions was previously defined by Peterson *et al*, (29), which included NuSAP1. GLI1 is involved in the intracellular signal transduction controlled by the Hedgehog family of secreted molecules (30). The current study further investigated whether the Hedgehog (HH)/Gli pathway was associated with NuSAP1. The results revealed no marked alteration in GLI1 mRNA expression levels following NuSAP1 knockdown in the HepG2 cell line (Fig. 6A), which suggested that NuSAP1 may not regulate the biological function of liver cancer through the HH/Gli pathway. NuSAP1 influences the DNA damage response by controlling breast cancer type 1 susceptibility protein (BRCA1) expression levels, and global DNA methylation is affected by BRCA1 through the regulation of DNMT1 (31,32). Thus, the expression of DNMT1 was also detected by RT-qPCR. The results revealed that NuSAP1 silencing significantly inhibited the mRNA level of DNMT1 in HepG2 cells compared with the control group (Fig. 6B).

Discussion

NuSAP1 is a microtubule and chromatin-binding protein that serves a role in spindle assembly (33). When NuSAP1 is overexpressed, microtubules aggregate into bundles (4,6).

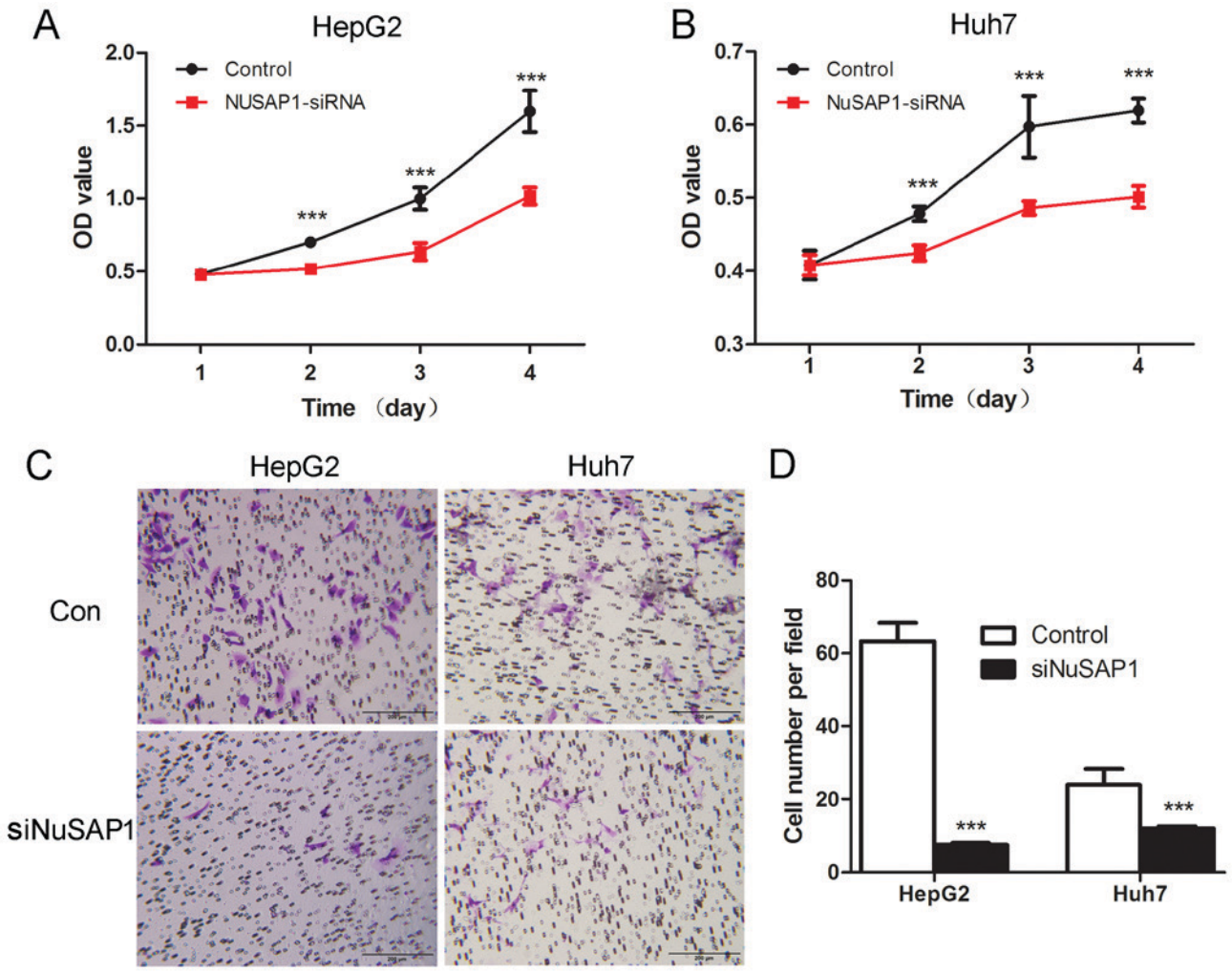

Figure 4. Effects of NuSAP1 silencing on HepG2 and Huh-7 cell proliferation and invasion. The effect of NuSAP1 knockdown on (A) HepG2 and (B) Huh-7 cell proliferation was evaluated by the Cell Counting Kit-8 assay, indicating that NuSAP1 knockdown inhibited cell growth after siNuSAP1 transfection for 72 h. (C) Representative micrographs of the Transwell invasion assay in HepG2 and Huh-7 cells transfected with siNuSAP1. Scale bar, 200 μm. (D) Knockdown of NuSAP1 suppressed the HepG2 and Huh-7 cell invasive potential compared with the control group. The error bar indicates the SEM. ***P<0.001 vs. the control group. NuSAP1, nucleolar and spindle-associated protein 1.

NuSAP1 deficiency in mice causes early embryonic lethality (34). The upregulation of NuSAP1 has been reported in a number of different human malignancies; however, few studies have demonstrated the altered expression of NuSAP1 in liver cancer (10-14,16,19). Therefore, to clarify the roles of NuSAP1 in liver cancer, the expression and biological functions of this gene were investigated. RT-qPCR and western blotting detected NuSAP1 protein expression in HCC tumor and adjacent tissues. The results revealed that NuSAP1 was upregulated in HCC tumor tissues, which was in accordance with a previous study (15). This result indicated that NuSAP1 is closely associated with HCC and may be used as a biomarker. Subsequently, the expression of NuSAP1 was determined in different HCC cell lines by RT-qPCR. The results revealed that NuSAP1 was highly expressed in HepG2 and Huh-7 cell lines. Although there was no significant alteration in NuSAP1 expression levels in SNU-423, compared with the normal human hepatic cell line LO2, SNU-423 may be a suitable cell line for overexpression of NuSAP1 to validate the function of this gene in future studies.

After years of optimization and improvements, lentiviral packaging is currently a mature genetic engineering technology applied in numerous studies (35,36). In the present study, NuSAP1 gene expression was effectively suppressed in the HepG2 and Huh-7 cell lines by lentivirus-mediated siRNA. Proliferation is not only necessary for the development and tissue homeostasis during adult life, it is also a hallmark of malignancy (37). To identify genes associated with the clinical outcome of breast cancer, NuSAP1 was detected in four independent studies (38). Therefore, in addition to being a reliable proliferation marker, NuSAP1 is a diagnostic marker for breast cancer and, potentially, other tumor types (38). The current data revealed that cell proliferation and invasion were significantly decreased following silencing of NuSAP1, indicating that its overexpression may enhance cell activity.

Cell cycle assays were performed to investigate the mechanism through which NuSAP1 promotes cell proliferation. The results revealed that the decrease in NuSAP1 expression in liver cancer cells delayed cell cycle progression by arresting the cycle at the G1 phase. It may be hypothesized that an

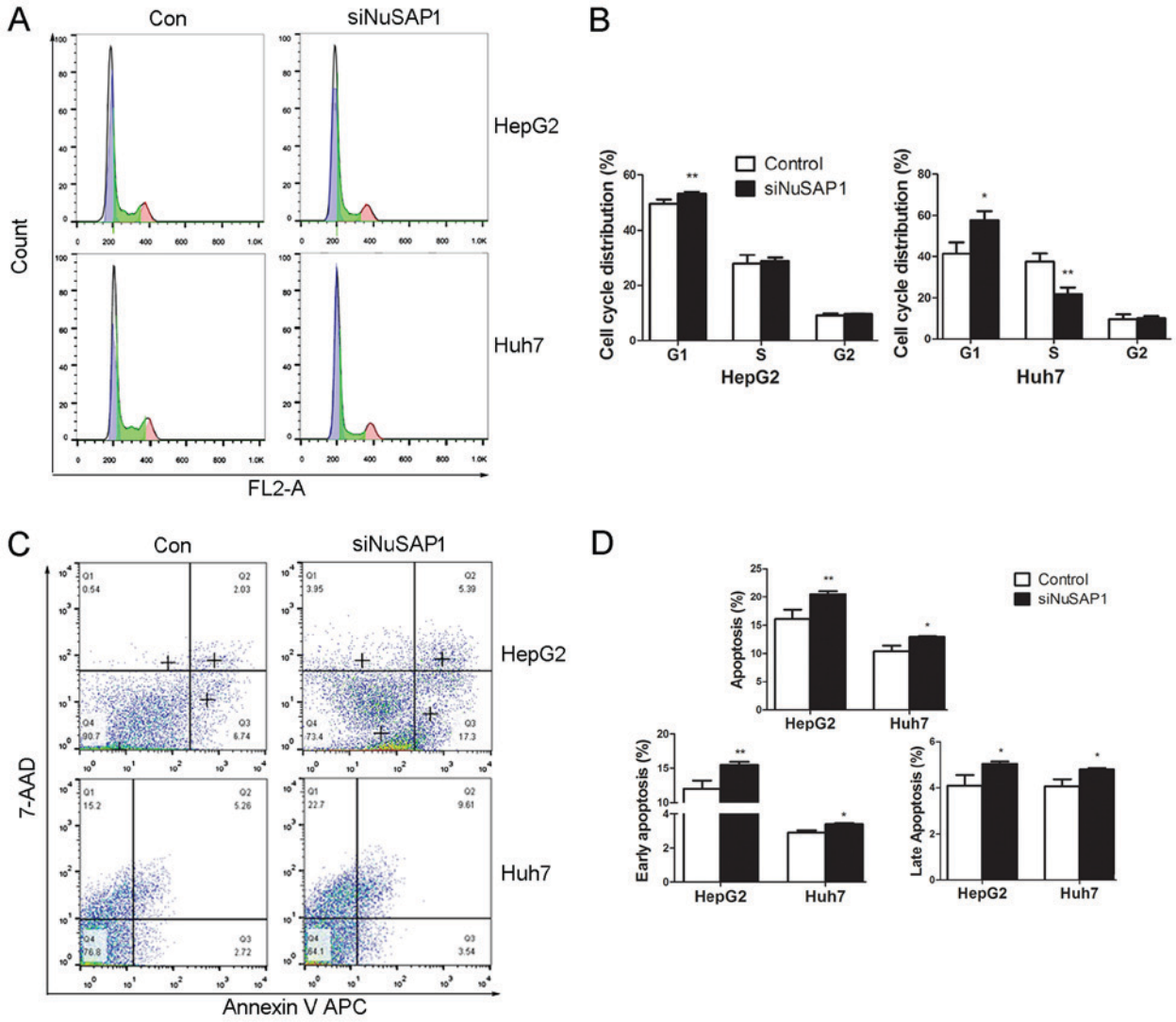

Figure 5. Effects of NuSAP1 silencing on HepG2 and Huh-7 cell cycle distribution and apoptosis. (A) Flow cytometric analysis of the cell cycle distribution in HepG2 and Huh-7 cells treated with siNuSAP1. (B) NuSAP1 knockdown increased cell cycle arrest at the G1-phase compared with the control group. (C) Flow cytometric analysis of the apoptotic rate in HepG2 and Huh-7 cells following siNuSAP1 treatment for 72 h. (D) NuSAP1 knockdown increased cell apoptosis compared with the control group. *P<0.05 and **P<0.01 vs. with the control. NuSAP1, nucleolar and spindle-associated protein 1; si, small interfering RNA; APC, allophycocyanin; 7-AAD, 7-aminoactinomycin D.

upregulated NuSAP1 expression induces a dysregulated cell cycle, which leads to the abnormal proliferation of tumor cells. Therefore, the accelerated liver cancer cell proliferation may be inhibited due to the decreased expression of NuSAP1.

A markedly increased apoptosis in NuSAP1-silenced liver cancer cells was observed in the current study. The mechanisms of apoptosis caused by the knockdown of NuSAP1 remain to be elucidated. The present results indicated that NuSAP1 serves a role in maintaining liver cancer cell growth. These data strongly suggest that NuSAP1 may affects tumor cell proliferation and apoptosis, which contributes to the pathogenesis of liver cancer.

A previous study revealed that NUSAP1 promotes invasion, migration and metastasis by modulating family with sequence similarity 101 member B, a transforming growth factor b1 signaling effector involved in the epithelial to mesenchymal transition (39). Another study indicated a strong correlation between E2F1 and NuSAP expression in human prostate cancer samples (16). In the present study, to further investigate the underlying molecular mechanism of NuSAP1 in liver cancer, the GLI1 and DNMT1 expression levels were measured in liver cancer cells using RT-qPCR. It has been previously reported that NuSAP1 promoted the aggressiveness of astrocytoma by activating the HH signaling pathway (18). However, no significant difference was observed in the GLI1 gene expression level following NuSAP1 silencing in the current study. A previous study revealed that NuSAP1 influenced the DNA damage response by controlling breast cancer type 1 susceptibility protein (BRCA1) expression levels (31). Global DNA methylation is affected by BRCA1 through the regulation of DNMT1 (32). Therefore, determining whether NuSAP1 may regulate the DNMT1 expression would be noteworthy. The present results indicated that NuSAP1 silencing inhibited the expression of the DNMT1 gene in liver cancer cells. However, the underlying mechanism remains unclear and requires further research. Overall, these results suggested that NuSAP1 silencing may have no effect on the HH signaling pathway.

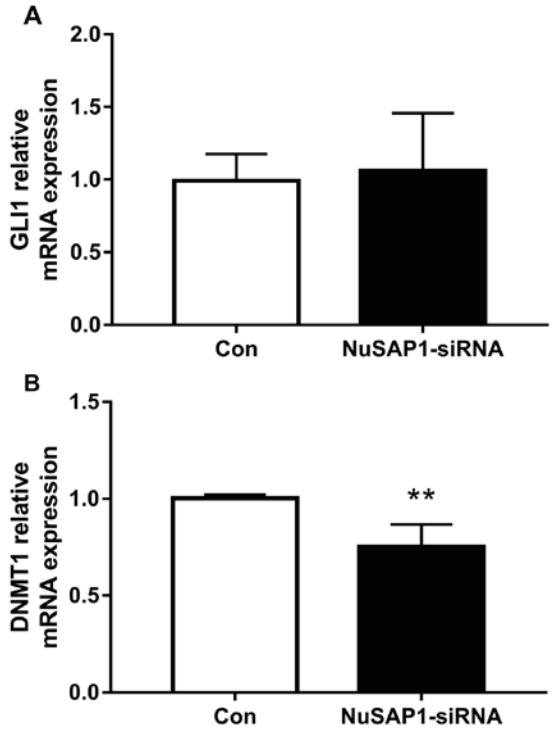

Figure 6. NuSAP1 silencing inhibits the DNMT1 but not GLI1 gene expression in liver cancer cell. (A) The relative GLI1 mRNA level was determined by RT-qPCR inHepG2 cells following treatment with siNuSAP1 for 72 h. β-actin was used as the internal control. No significant difference in the GLI1 mRNA level was observed following NuSAP1 knockdown. (B) The relative DNMT1 mRNA level was determined by RT-qPCR in HepG2 cells following treatment with siNuSAP1 for 72 h. β-actin was used as an internal control. NuSAP1 knockdown significantly decreased the mRNA level of DNMT1. **P<0.01 vs. the control. NuSAP1, nucleolar and spindle-associated protein 1; DNMT1, DNA methyltransferase; GLI1, glioma-associated oncogene; RT-qPCR, reverse transcription quantitative polymerase chain reaction; si, small interfering RNA.

However, NuSAP1 silencing inhibited the DNMT1 gene expression in liver cancer cells.

In conclusion, the current study demonstrated that NuSAP1 upregulation may be an early event in the processes of liver cancer, therefore NuSAP1 may serve an important role in liver cancer development. NuSAP1 may provide a new target for liver cancer gene therapy. Although the underlying mechanisms require further investigation, the current study provides an improved understanding of the function of NuSAP1 in liver cancer and lays the foundation for the development of novel liver cancer treatment strategies.

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Availability of data and materials

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

Authors' contributions

ZB and ZL contributed to the study design and literature search. YW contributed to the western blotting, immunohistochemistry, RT-qCPR, writing of the manuscript, statistical analysis and figure creation. FX contributed to the tissue collection and histopathological analysis. HL and LJ performed the flow cytometry. XL and LC contributed to the data collection.

Ethical approval and consent to participate

The current study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The current study was reviewed and approved by the Ethics Committee of the Nantong Third People's Hospital Affiliated to Nantong University.

Patient consent for publication

Informed consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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