



Nucleolar spindle associated protein 1 (NUSAP1) facilitates proliferation of hepatocellular carcinoma cells

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Background: The most fundamental characteristic of cancer cells is abnormal proliferation, which is partly due to deregulation of cell cycle. Nucleolar spindle associated protein 1 (NUSAP1) is originally identified as microtubule and chromosome binding protein and takes an important role in chromosome segregation fidelity. Recent studies have been documented that NUSAP1 is associated with the progression of several cancers. However, its expressions and functions are unclear in hepatocellular carcinoma (HCC).

Methods: qPCR and western blotting were used to examine NUSAP1 levels in fresh paired HCC tissues and corresponding adjacent normal liver tissues (ANT). MTT and colony formation assay were performed to evaluate whether NUSAP1 can influence the proliferation of HCC cells. Anchorage-independent growth assay was implemented to detect the effect of NUSAP1 on *in vitro* tumorigenicity capacity. Flow cytometry, qPCR and western blotting assay were used to clarify the detailed mechanisms that NUSAP1 accelerates the capability for cell proliferation and *in vitro* tumorigenicity.

Results: Firstly, we analyzed the available public dataset The Cancer Genome Atlas (TCGA), and discovered that NUSAP1 is dramatically upregulated in HCC tissues and closely correlated with poor prognosis of patients suffering HCC, which was confirmed in fresh HCC tissues and ANT. Subsequently, cell function assay illustrated that the upregulation of NUSAP1 facilitates proliferation and *in vitro* tumorigenicity capacity of HCC cells. Besides, molecular experiments suggested that NUSAP1 promotes cell cycle transition of HCC cells.

Conclusions: In summary, the above results inferred that NUSAP1 takes an indispensable role in cell cycle progression. And it might be served as a novel prognostic factor in HCC.

Keywords: Cell cycle transition; hepatocellular carcinoma (HCC); proliferation; nucleolar spindle associated protein 1 (NUSAP1)

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Introduction

Liver cancer is evaluated to be the sixth most commonly diagnosed cancer and the fourth leading reason of cancer related death worldwide in 2018, with approximate 841,000

new cases and 782,000 deaths yearly. Among liver cancer, 78–85% of cases are hepatocellular carcinoma (HCC) (1). Therefore, it is essential to investigate the detailed mechanisms of tumorigenicity in HCC.

The most fundamental characteristic of cancer cells

is abnormal proliferation (2), which is partly caused by misregulation of cell cycle. The cell cycle of eukaryotic cells includes four phase, namely, G1, S, G2 and M. The phase transition is critically driven by cyclin-dependent kinases (CDKs). As the central regulatory determinant in cell cycle, CDKs are suffered to many levels of regulation, and their activities change dependent on both intracellular and extracellular environment (3,4). The first level of regulation is implemented by cyclins, and another level is by CDKs inhibitors (CKIs) to inhibit phosphorylation of CDKs. Of which, cyclin abundance oscillates during the cell cycle as a result of programmed synthesis and degradation. The G1/S phase transition is driven by CDK2, CDK4, CDK6 and corresponding cyclins. CDK4 and CDK6 are regulated by D-type cyclins: cyclin D1, cyclin D2 and cyclin D3. Cyclin E accumulates very closely to G1/S transition and specifically activates CDK2. Activated CDKs can target and phosphorylate retinoblastoma (p-Rb). p-Rb is identified to be the first suppressor gene. It can repress transcription through binding transactivation domain of E2F family (5). Phosphorylated p-Rb alleviates its inhibition on E2F family and allows the transcription of genes essential for S phase entry and DNA synthesis (6). CDK1 or CDK2 together with cyclin A are indispensable for the continuation of S phase and entry into M phase. CDK1 together with cyclin B also promote entry into M phase, and cyclin A is degraded before cyclin B by the proteasome (7). The intracellular and extracellular signals that affect phase transition of cell cycle play crucial roles in cell cycle progression. Therefore, we aim to investigate the factors that affect the cell cycle transition.

Nucleolar spindle associated protein 1 (NUSAP1) is originally identified as microtubule and chromosome binding protein. It takes an important role in chromosome segregation fidelity (8-10). Recently, it has been documented that NUSAP1 is abnormal expression, which is associated with poor prognosis in astrocytoma (10), prostate cancer (11), oral squamous cell carcinoma (12), prostate cancer (13), and breast cancer (14). However, its expressions and functions are unclear in HCC.

Herein, we found the expression of NUSAP1 significantly elevates and is associated with poor prognosis in HCC. Further assay suggested that upregulation of NUSAP1 can promote cell cycle progression of HCC cells. The above results demonstrate that NUSAP1 might be a prognostic marker for HCC.

Methods

Cell line and tissue specimens

HCC cell HepG2 was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured using Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) that contained 10% fetal bovine serum (FBS) (HyClone, UT, USA). The primary HCC tissues and ANT were collected from Sun Yat-sen Memorial Hospital. For research purpose, we acquired the informed consent of patient involved in the research. This study was approved by institutional ethics committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (No. W2017NJ07). The research has been carried out in accordance with the Declaration of Helsinki [2008] of the World Medical Association.

Plasmids and retroviral infection

For overexpression of NUSAP1, the full-length human NUSAP1 cDNA was amplified and cloned into the pMSCV vector. For downregulation of NUSAP1, 2 human siRNA sequences that target NUSAP1 were sub-cloned into pSuper-retro-puro plasmid. And the siRNA fragments are as follows: Sh#1: GAGCACCAAGAAGCTGAGAAT; Sh#2: GAACCACACAAAGGAAAGCTA. Infection was conducted using retrovirus. To get stable cell lines, we treated the cells using 0.5 µg/mL puromycin. 10 days later, we detected the levels of NUSAP1 in cells.

Quantitative PCR (qPCR)

We used the Trizol reagent (Invitrogen, Carlsbad, CA, USA) to collect total RNA. qPCR was performed using ABI 7500 Fast System (Applied Biosystems, Rockville, MD, USA). We evaluated the relative amount of each gene on the basis of the algorithm: $2^{-[(Ct \text{ of gene}) - (Ct \text{ of } \beta\text{-Tubulin})]}$. In the algorithm, the Ct is defined as threshold cycle and we used β -Tubulin as the internal control. The following are the primers of genes: NUSAP1: forward, 5'-CTGACCAAGACTCCAGCCAGAA-3', reverse, 5'-GAGTCTGCGTTGCCTCAGTTGT-3'; Cyclin D1: forward, 5'-TCTACACCGACAACCTCCATCCG-3', reverse, 5'-TCTGGCATTTTGGAGAGGAAGTG-3'; Cyclin D3: forward, 5'-AGATCAAGCCGCACATGCGGAA-3', reverse, 5'-ACGCAAGACAGGTAGCGATCCA-3'; Cyclin E1:

forward, 5'-TGTGTCCTGGATGTTGACTGCC-3', reverse, 5'-CTCTATGTGCGACCACTGATACC-3'; Cyclin A2: forward, 5'-CTCTACACAGTCACGGGACAAAG-3', reverse, 5'-CTGTGGTGCTTTGAGGTAGGTC-3'; Cyclin B1, forward, 5'-GACCTGTGTCAGGCTTTCTCTG-3', reverse, 5'-GGTATTTTGGTCTGACTGCTTGC-3'.

Western blotting assay

Western blotting assay was carried out on the basis of methods described (15). Whereafter, the membrane was probed by the primary antibodies: anti-NUSAP1, anti-p-Rb, anti- β -Tubulin (Abcam, Cambridge, MA, USA), and subsequently probed using HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA). And we used β -Tubulin as the loading control.

3-(4,5-Dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

2×10^3 cells were seeded into 96-well plates, subsequently maintained in 37 °C incubator until indicated time. Whereafter, the cells were treated by 100 μ L 0.5 mg/mL sterile MTT reagent (sigma, St Louis, MO, USA) for 4 hours. And then, we added 150 μ L dimethyl sulphoxide (DMSO) (sigma, St Louis, MO, USA) into the plates. And the absorbance of cells was detected at 490 nm.

Colony formation assay

Colony formation was used to evaluate the effect of NUSAP1 on proliferation of HCC cells and carried out on the basis of the methods described (16). 5×10^2 cells were implanted into 6-well plates and maintained in 37 °C incubator for 10 days. Firstly, we used 10% formaldehyde to fix the colonies. And then, 1% crystal violet was applied to stain the colonies for 30 seconds.

Anchorage-independent growth assay

The completed medium that contains 1% agar (sigma, St Louis, MO, USA) was supplemented into the bottom of the cell plates. Then, the mixture that was composed of 500 cells, 0.3% agar and 2 mL complete medium were added into the upper of the plates. The cells were maintained in 37 °C incubator for 10 days and then the colonies with diameter larger than 0.1 mm were counted.

Flow cytometry assay

We collected the cells and fixed with 75% ethanol. Then, we applied 20 μ g/mL RNase A and 50 μ g/mL propidium iodide to treat the cells for 30 minutes away from light. The cells-treated were detected using FASCS alibur instrument (BD Biosciences, Franklin Lake, New Jersey, USA). Besides, CellQuest 3.3 software was used to evaluate the cells percentages in different phases.

Statistical analysis

Every experiment was repeated at least 3 times. SPSS 20.0 statistical software package was used to perform statistical analysis. The statistical significance between groups was evaluated by Student's *t*-test, and the data was demonstrated as mean \pm deviation. Survival curves were plotted using the Kaplan-Meier method, and significant analysis was using log-rank test. $P < 0.05$ was considered as statistical significance.

Results

NUSAP1 is significantly upregulated and associated with poor survival in HCC

Through analyzing the available public dataset The Cancer Genome Atlas (TCGA) (<https://cancergenome.nih.gov/>), the results showed that NUSAP1 expression is significantly upregulated in 351 HCC tissues compared with 50 normal liver tissues (*Figure 1A*). Subsequently, we further evaluated the NUSAP1 expression in 50 paired tissues. As demonstrated in *Figure 1B*, NUSAP1 is drastically upregulated in 47 HCC tissues compared with matched adjacent normal liver tissues (ANT). Besides, the correlation between NUSAP1 expression and clinical pathologic characteristics were performed. NUSAP1 expression is significantly upregulated in AJCC stage III-IV compared with that in stage I-II (*Figure 1C*). Not only that, the patients with high-expression of NUSAP1 showed poorer 5-year overall survival and relapse-free survival than that with low NUSAP1 (*Figure 1D*).

We also check the NUSAP1 expression in 8 paired fresh HCC tissues and ANT using qPCR and western blotting assay. The results demonstrated that NUSAP1 expression is drastically elevated in HCC tissues than in ANT both on transcription and translation levels (*Figure 2*).

Altogether, NUSAP1 is significantly upregulated in HCC tissues and associated with poor prognosis.

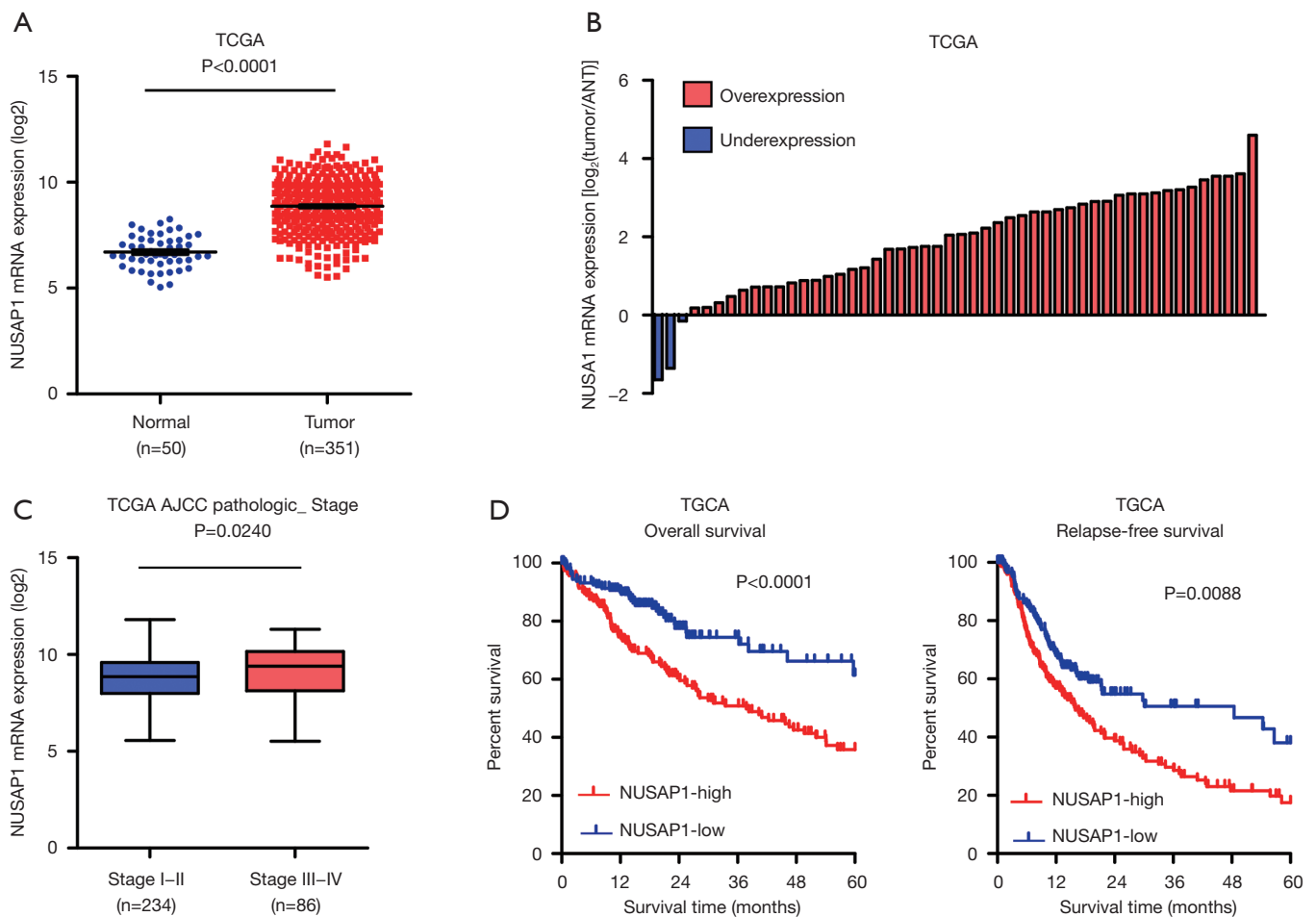


Figure 1 NUSAP1 expression is significantly upregulated in HCC by analyzing TCGA. (A) The NUSAP1 mRNA expression is drastically upregulated in HCC tissues compared with normal liver tissues. (B) The NUSAP1 mRNA expression in 50 paired tissues. (C) NUSAP1 mRNA expression is significantly upregulated in HCC stage III-IV compared with in stage I-II. (D) Kaplan-Meier 5-year overall survival (left panel) and relapse-free survival (right panel) curves for patients with high and low NUSAP1. NUSAP1, nucleolar spindle associated protein 1; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

Overexpression of NUSAP1 promotes the proliferation and in vitro tumorigenicity capacity of HCC cells

To investigate the functions of NUSAP1 in HCC progression, the gene set enrichment analysis (GSEA) was carried out using the data from TCGA. The analysis illustrated that NUSAP1 might be involved in cell cycle progression (Figure 3A). Next, the NUSAP1-overexpressed and -silenced stable cell lines were screened using HCC cell HepG2. The mRNA and protein expression of NUSAP1 was examined in the stable cell lines (Figure 3B). Next, MTT and colony formation assay demonstrated that overexpression of NUSAP1 significantly promoted HCC cells proliferation, and knockdown of NUSAP1 drastically

inhibited cell proliferation (Figure 3C,D). Besides, anchorage-independent growth assay illustrated that *in vitro* tumorigenicity capacity of HCC cells promotes in NUSAP1-upregulating cells, while inhibits in NUSAP1-downregulating cells (Figure 3E).

Altogether, overexpression of NUSAP1 promoted proliferation and *in vitro* tumorigenicity capacity of HCC cells.

NUSAP1 facilitates cell cycle transition in HCC cells

To further explore the mechanisms by which NUSAP1 promotes cell proliferation and *in vitro* tumorigenicity capacity, GSEA was implemented. The analysis

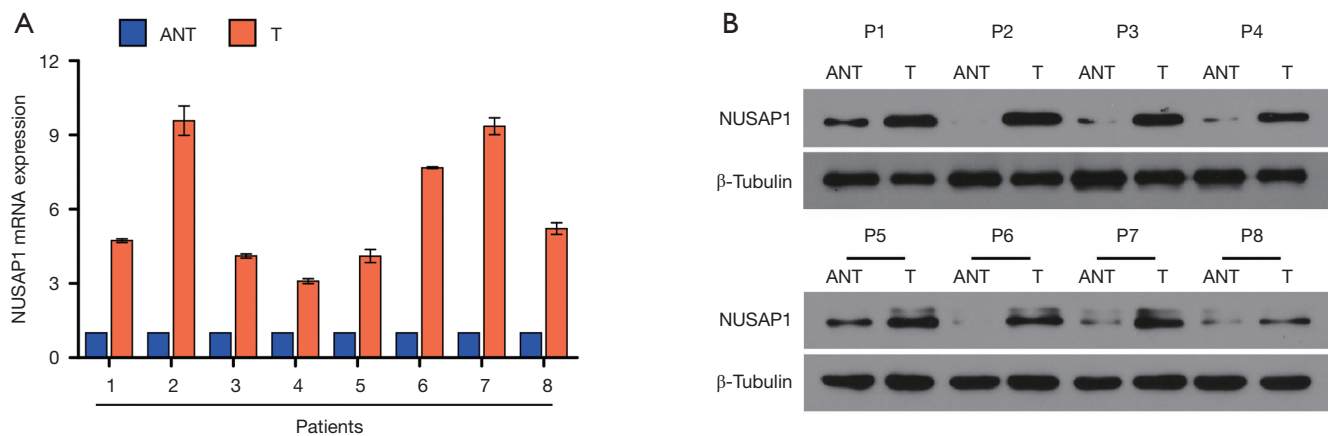


Figure 2 NUSAP1 expression is drastically upregulated in fresh HCC tissues compared with ANT by qPCR (A) and Western blotting assay (B). NUSAP1, nucleolar spindle associated protein 1; HCC, hepatocellular carcinoma; ANT, adjacent normal liver tissues.

demonstrated that NUSAP1 might be involved in G1/S and G2/M phase transition (Figure 4A). Next, flow cytometric assay was performed. As shown in Figure 4B, overexpression of NUSAP1 increases the percentage of cells in S-phase, but decreases cells in G0/G1 phase. While, downregulation of NUSAP1 showed the opposite results (Figure 4B). Not only that, we evaluated the mRNA expression of cyclins involved in G1/S and G2/M phase transition, cyclin D1, cyclin D3, cyclin E1, cyclin A2, cyclin B1 and the expression of E1F2. The heatmap illustrated that overexpression of NUSAP1 significantly increases their expression and downregulation of NUSAP1 inhibits their expression (Figure 4C). Besides, the phosphorylation level of p-Rb is the critical determinant of G1/S phase transition. Therefore, we evaluated the phosphorylation level of p-Rb by western blotting assay. As shown in Figure 4D, the phosphorylation level of p-Rb enhances in NUSAP1-upregulated cells, while downregulates in NUSAP1-silenced cells, indicating that NUSAP1 can help to G1/S transition.

Altogether, overexpression facilitates cell cycle G1/S and G2/M phase transition of HCC cells.

Discussion

Herein, we discovered that the level of NUSAP1 dramatically elevates in HCC tissues and is negatively related with prognosis of patients with HCC. Further analysis demonstrated that upregulation of NUSAP1 promotes the proliferation and *in vitro* tumorigenicity capacity of HCC cells and facilitates the G1/S and G2/M phase transition.

Aberrant proliferation of cells results in loss of the normal

tissue architecture and functions, and the cancer cells obtain the capability for aberrant proliferation. Aberrant proliferation can be caused by multiple intracellular and extracellular signals. It has been reported that lncRNA FAL1 is up-regulated in HCC tissues and accelerates cell proliferation (17). Lin *et al.* showed that Exportin-T can promote cell proliferation through affecting cell cycle and ubiquitin-mediated proteolysis of HCC cells (18). Liu *et al.* clarified that PNMA1 significantly elevates in HCC tissues and is associated with poor prognosis (19). Shi and her colleagues suggested that TNNT1 is significant upregulated in breast cancer cells, and upregulation of TNNT1 can promote G1/S transition of breast cancer cells. Especially, TNNT1 can increase the levels of cyclin D1, cyclin E1, cyclin E2 and the phosphorylation of Rb (16). In particular, in consideration of CDKs ultimately driving the cell cycle transition, substantial preclinical test on CKIs are underway. Whereas, the results of clinical trials are disappointing (20). Our study might provide a novel idea on targeting cell cycle. NUSAP1 is upregulated to accelerate cell proliferation in HCC and essential for the integrity of the anaphase spindle and cell division. We speculated that targeting NUSAP1 can lead to abnormal cell division, which might be result in cell apoptosis.

Wu *et al.* discovered that NUSAP1 also promotes cancer metastasis by activating the Hedgehog signaling pathway in astrocytoma (21). Gordon and his colleagues found that NUSAP1 enhances invasion and metastasis in prostate cancer (11). Whereas, whether NUSAP1 also promotes metastasis in HCC is unclear. We will clarify the above problems in our future study.

In conclusion, NUSAP1 is significantly upregulated in

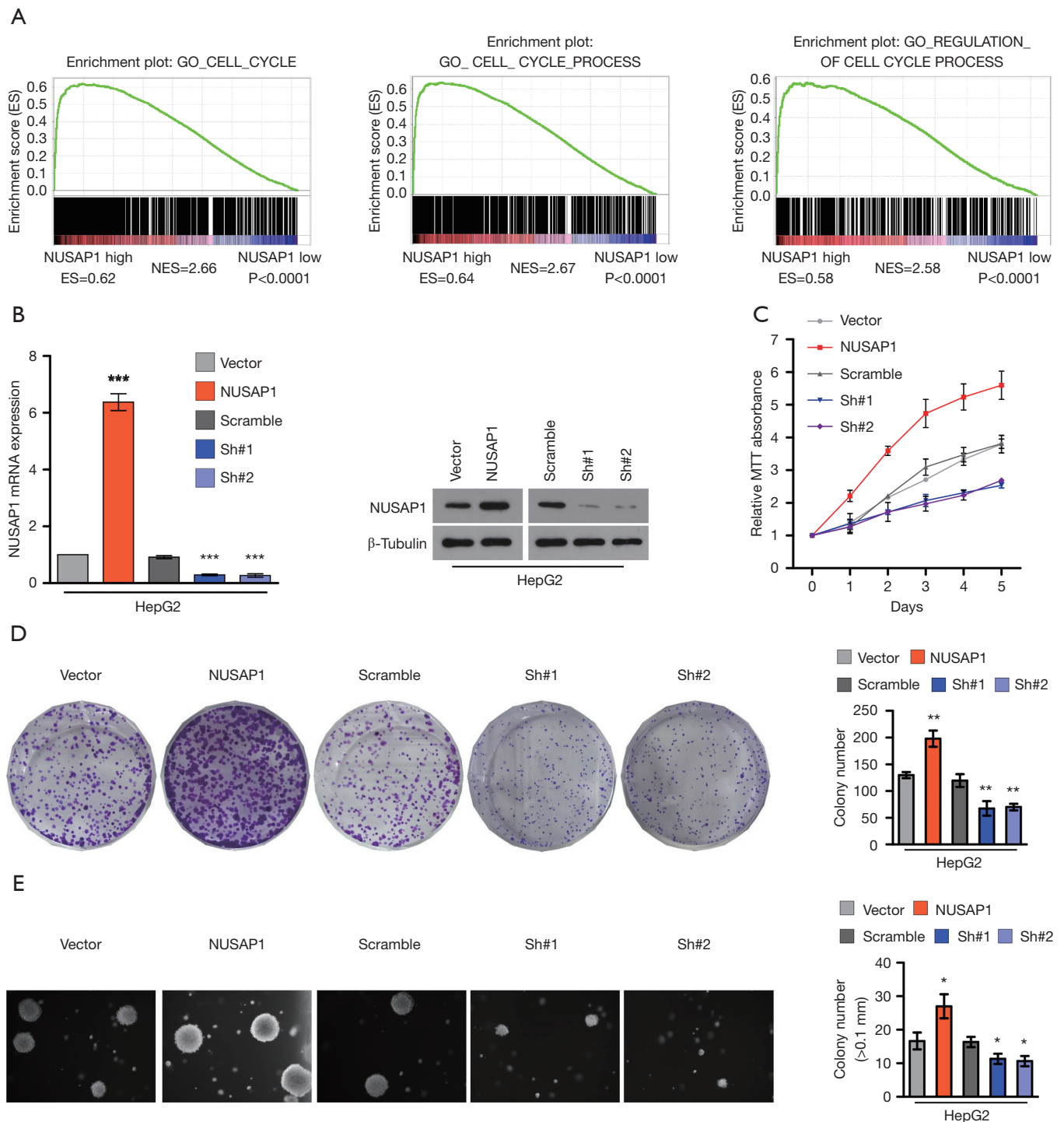


Figure 3 NUSAP1 can promote proliferation and *in vitro* tumorigenicity of HCC cells. (A) Gene set enrichment analysis (GSEA) illustrated that NUSAP1 might be involved in cell cycle progression using analyzing TCGA data. (B) qPCR (left panel) and western blotting assay (right panel) in indicated stable cell lines. (C) MTT assay showed that overexpression of NUSAP1 significantly promotes, while knockdown inhibits the proliferation of HCC cells. (D) Colony formation assay were used to detect the effect of NUSAP1 on HCC cell proliferation. (E) Anchorage-independent growth ability assay were performed to examine the effect of NUSAP1 on *in vitro* tumorigenicity of HCC cells. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$. NUSAP1, nucleolar spindle associated protein 1; HCC, hepatocellular carcinoma.

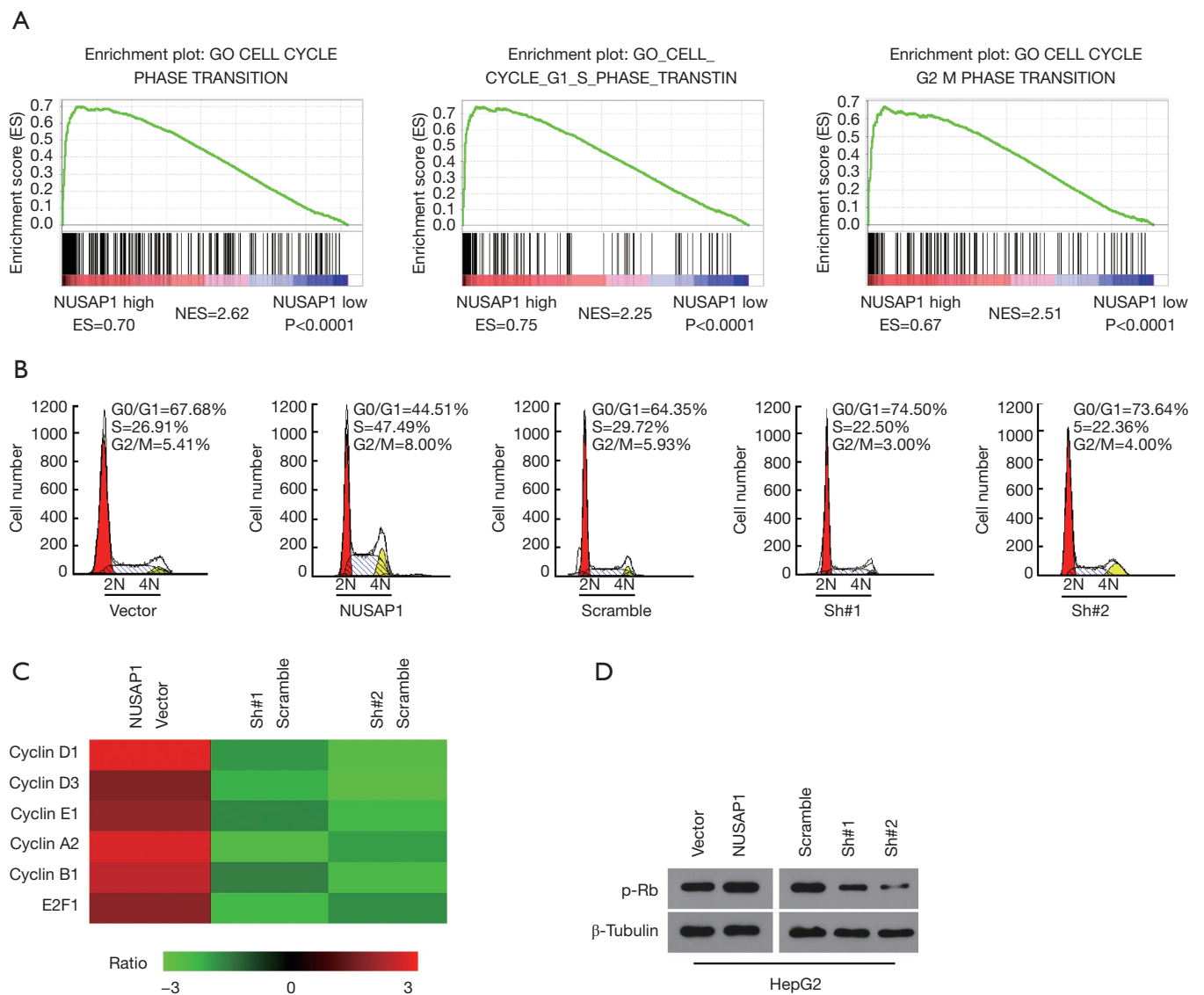


Figure 4 Overexpression of NUSAP1 facilitates cell cycle transition of HCC cells. (A) GSEA illustrated that NUSAP1 might be involved in G1/S phase and G2/M phase transition. (B) Flow cytometry assay demonstrated that overexpression of NUSAP1 increases the percentage of cells in S and G2/M phase and decreases in G0/G1 phase. (C) The heatmap for the expression of cyclin D1, cyclin D3, cyclin E1, cyclin A2, cyclin B1 and E1F2 in indicated cells. (D) The phosphorylation levels of p-Rb in indicated cells. NUSAP1, nucleolar spindle associated protein 1; HCC, hepatocellular carcinoma; GSEA, gene set enrichment analysis.

HCC and associated with poor prognosis of patients with HCC. Further assay showed that NUSAP1 can promote cell cycle progression. Our study might provide a novel therapeutic prognostic factor for HCC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org>).

[org/10.21037/tcr.2019.09.28](https://doi.org/10.21037/tcr.2019.09.28)). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. For research purpose, we acquired the informed consent of patient involved in the research. This study was approved by institutional ethics committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (No. W2017NJ07). The research has been carried out in accordance with the Declaration of Helsinki (as revised in 2013) of the World Medical Association.

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