Nucleostemin/GNL3 promotes nucleolar polyubiquitylation of p27kip1 to drive hepatocellular carcinoma progression

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Abstract

p27kip1, as a cyclin dependent kinase inhibitor (CDKI), plays a pivotal role in the regulation of cell cycle progression and hepatocarcinogenesis. Herein, we revealed that p27 exhibited apparent nucleolar distribution and interacted with nucleolar protein nucleostemin (NS) in Hepatocellular carcinoma (HCC) cells. Furthermore, subcellular fractionation experiments demonstrated that nucleolar p27 had significantly higher level of polyubiquitylation, compared with nucleoplasmic fraction. Depletion of NS inhibited nucleolar polyubiquitylation of p27, indicating an involvement of NS in triggering p27 ubiquitylation and inactivation during HCC development. Moreover, we found that knockdown of NS promoted p27 to bind to CDK2–Cyclin E complex and inhibited the activity of CDK2, resulting in consequent cell cycle arrest in HCC cells. Furthermore, silencing NS expression reduced in vitro colony formation and in vivo tumor growth of HCC cells. Finally, we found that NS was upregulated in HCC tissues, compared with adjacent non-tumorous tissues. Kaplan–Meier analysis indicated patients with high expression of NS and low expression of p27 had significantly worsened prognosis. Our results suggested NS mediated p27-dependent cell cycle control via inducing nucleolar sequestration and polyubiquitylation of p27 in HCC. These findings help gain an insightful view into the mechanism underlying aberrant cell cycle progression during hepatocarcinogenesis, and thus benefit the development of molecular-targeted therapies in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and is the third leading cause of cancer-related mortality worldwide [1]. Liver resection and liver transplantation remain the most widely applicable treatment for HCC patients. However, the prognosis for advanced HCC remains dismal, with a low 5-year survival rate of approximately 10% [2]. Until now, the molecular mechanisms underlying HCC development have not been fully understood, limiting the exploration of HCC therapy. Aberrant cell cycle progression and malignant proliferation are the common mechanisms underlying HCC development [3]. Therefore, clarifying the molecular mechanisms underlying HCC cell cycle progression and exploring corresponding therapeutic strategies could be of great value for increasing the chance of survival and improving the quality of life for patients with HCC.

p27kip1, a member of the cyclin-dependent kinase inhibitors (CDKIs) family, plays a pivotal role in cell cycle regulation and tumorigenesis [4]. CDKIs tightly control the timing and extent of CDK activation and reversibly halt cell cycle proliferation [5]. p27 primarily binds to and inhibits the activity of CDK2–Cyclin A/E and CDK4–Cyclin D complexes to prevent premature G1/S-phase transition during cell cycle control and tumor development [6]. Previous studies have shown that p27 loss-of-function resulted in uncontrolled proliferation of tumor cells [7]. Recent reports indicated that abnormal localization of p27 also plays a vital role in p27 function and tumor cell proliferation. For example, threonine 157 is phosphorylated by Akt and causes retention of p27 in the...
cytoplasm, precluding p27-induced G1 arrest and promoting breast cancer cell proliferation [8]. Nuclear export protein CRM1 promotes p27 translocation from nucleus to cytoplasm, resulting in p27 inactivation and epithelial ovarian cancer cell proliferation [9]. Furthermore, our recent research revealed that MIF4G6 maintained the stability of p27 both in the nucleus and cytoplasm and inhibited HCC cell proliferation [10]. Therefore, both the expression and subcellular distribution of p27 affect its role in tumor biology. However, the molecular mechanisms underlying p27 inactivation in HCC remain poorly understood and need further investigations.

Our previous researches focused on the expression of p27 in hepatocarcinogenesis [10–12]. Interestingly, we identified that the immunofluorescent signal of p27 highly resembled nuclear distribution in HCC tissues and cells. Combined with the fact that nucleoplasmic localization is a prerequisite for p27 to function as a cell cycle regulator [13], we speculated that certain factors may cause nucleolar distribution and influence p27 activity, which eventually contributes to hepatocarcinogenesis. Using immunoprecipitation-mass spectrometry (IP-MS) analysis, we identified nucleostemin/GNL3, designated as NS, as a novel binding protein of p27. NS was initially identified as a nucleolar protein that was highly expressed in neural stem cells, embryonic stem cells and cancer cells, and was absent in differentiated somatic cells [14–16]. Later studies reported that NS was essential for cell cycle regulation and cell proliferation in various human cancers, including liver cancer, gastric cancer and other malignancies [15,17,18]. Furthermore, studies also demonstrated that NS could recruit their interactive proteins to nucleolus and alter the function of the downstream molecules. For example, mobilization of NS recruits MDM2 to the nucleolus, maintains MDM2 protein stability and suppresses p53 activity in tumor cells [19]. Likewise, NS is able to form stable protein complex with alternative reading frame (ARF) protein in the nucleolus, which prevents ARF from interacting with its E3 ligase ULF in the nucleoplasm and subsequently inhibits the ubiquitylation and proteasomal degradation of ARF [20]. More importantly, Yoshida et al. revealed that depletion of NS upregulated p27 level and inhibited cell proliferation in oral squamous cell carcinomas, suggesting that NS expression may contribute to p27 downregulation during tumor development [21]. Given the fact that high expression of NS was detected in HCC cells and contributed to HCC cell proliferation, we speculated that NS protein might induce nucleolar inactivation of p27 through direct NS–p27 association, leading to the hyperactivation of CDK2 and resultant cell cycle progression [22].

Materials and methods

Cell lines and antibodies

The human embryonic kidney 293T cells, HCC cell lines (HepG2, Hep3B, Huh7, SMCC-7721, SK-Hep1) and normal liver cell line LO2 were obtained from Shanghai Institute of Cell Biology, Academic Sinica. The supplier performed cell line authentication. Cell lines and antibodies

Materials and methods

Cell proliferation assay and colony formation assay

Western blot and immunoprecipitation

Western blot analysis was conducted as previously described [24]. Briefly, the frozen liver tissues and harvested cells were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl (pH 7.4), 1% Nonidet P-40, 1 mM EDTA, 1 mM protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and centrifuged at 4 °C, 13,000 g for 15 min. After centrifugation, the supernatant, the protein concentration was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). The protein samples were sonicated for 8 × 10 bursts using a 450 Branson Sonifier at power setting 4. Later on, the sonicated samples were layered over with 0.5 ml S3 (0.88 M Sucrose, 0.5 mM MgCl2) and centrifuged for 10 min at 3000 g. The supernatants were collected as the nucleoplasmic fraction. The pellets were resuspended with 0.5 ml of S2 solution and centrifuged for 5 min at 14300 g to acquire highly purified nucleoli.

Subcellular fractionation

Cell fractionation to isolate nucleoli was performed as a previous report [23]. Briefly, 5 × 106 cells were harvested by trypsinization, washed three times with ice-cold PBS, resuspended in 1.5 ml Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT) and incubated on ice for 5 min. Thereafter, the lysate samples were transferred into a Dounce homogenizer to homogenize for 30 times, and immediately centrifuged for 5 min at 218 g, 4 °C. Next, the pellets were resuspended with 0.5 ml S1 solution (0.25 M Sucrose, 10 mM MgCl2) and centrifuged 5 min at 14300 g. Then, the pellets were collected and resuspended with 0.5 ml S2 solution (0.35 M Sucrose, 0.5 mM MgCl2). The samples were sonicated for 8 × 10 bursts using a 450 Branson Sonifier at power setting 4. Later on, the sonicated samples were layered over with 0.5 ml S3 (0.88 M Sucrose, 0.5 mM MgCl2) and centrifuged for 10 min at 14300 g. The pellets were collected as the nuclear fraction. The pellets were resuspended with 0.5 ml of S2 solution and centrifuged for 5 min at 14300 g to acquire highly purified nucleoli.
with the manufacturer's instructions. Each experiment was performed for three times at least.

For colony formation assay, the cells infected with control or NS-shRNA lentivirus were plated into a six-well plate (500 cells/well). 15 days later, the colonies were stained with 0.5% crystal violet for 30 min. Lastly, cell colonies (0.5 mm in diameter) were counted after staining.

Flow cytometric analysis

The cells were digested by 0.25% trypsin and fixed with 70% methanol for 2 days at –20 °C. Then, the cells were incubated with 200 μg/ml RNase A and 50 μg/ml propidium iodide (PI) (Becton Dickinson, San Jose, CA) at 37 °C for 30 min. The stained cells were analyzed by a fluorescence-activated cell sorter (FACS) Calibur (Becton–Dickinson, Franklin Lakes, NJ) and data were analyzed by CellQuest and Modfit software programs.

Xenograft mouse model

Fifteen 5-week-old female BALB/c nude mice from Shanghai SLAC Animal Center were used for the subcutaneous injection of cells from different group. A total of 2 x 10^6 cells were resuspended with 0.2 ml cold PBS and subcutaneously injected into the flank of nude mice. After injection, the size of the tumor was measured with a digital caliper every 5 days. The tumor volume was measured and calculated using the following formula: volume = length x width x height x 0.5. Forty days later, the mice were sacrificed and tumors were excised, measured and weighed. All animal experiments were performed under the approval of the Nantong University Animal Care Committee.

Immunohistochemical analysis

Immunohistochemical analysis was conducted as previously described [9]. Briefly, the FFPE tissue microarray was dewaxed in xylene and rehydrated in graded alcohol. Thereafter, the microarray was boiled in 0.01 M citrate buffer (pH 6.0) for 3 min in an autoclave to retrieve the antigen and subsequently incubated in hydrogen peroxide (0.3%) for 20 min at room temperature to block endogenous peroxidase activity. After washing three times with PBS, the tissue microarray was incubated with anti-NS antibody (1:100) and anti-p27 antibody (1:200) overnight at 4 °C. After washing three times, the tissues were incubated with horse-radish peroxidase-conjugated anti-rabbit or anti-mouse IgG as secondary antibodies (Envision kit, Dako) for 30 min at room temperature. Then, the microarray was counterstained with hematoxylin, dehydrated, and mounted in resin mount. Lastly, the stained microarray was examined under a microscope.

Statistical analysis

All statistical analysis was performed using the State 7.0 software package. The association between NS expression and clinicopathological features was analyzed using the χ² test. For analysis of survival data, Kaplan–Meier curves were constructed. P < 0.05 was considered to be statistically significant.

Additional and detailed methods are described in the Supplementary Information.

Results

NS interacts with p27 in vivo and in vitro

To gain a better insight into the mechanism underlying nuclear distribution of p27, we firstly examined whether p27 colocalized with fibrillarin, a well-characterized nucleolar marker protein, using immunofluorescent analysis in HCC cell. As shown in Supplementary Fig. S1, p27 was localized in the nucleoli but did not exhibit significant overlap with fibrillarin. This result indicated that nucleolar localization of p27 might involve some unknown nucleolar proteins. As such, we identified NS as a potential binding partner of p27 using immunoprecipitation-mass spectrometry (IP-MS) analysis. To verified the interaction between p27 and NS, Myc-tagged p27 (myc-p27) and Flag-tagged NS (Flag-NS) were co-transfected into HEK293T cells and then subjected to immunoprecipitation assay. As shown in Fig. 1A, p27 was brought down by an anti-Flag antibody from the 293T cell lysate. Next, the interaction between endogenous NS and p27 was verified through reciprocal immunoprecipitation with cell lysates from HepG2 that had high NS expression (Fig. 1B and Supplementary Fig. S2). Meanwhile, GST pull-down assay was performed to determine the direct association between GST-p27 and Flag-NS (Fig. 1C). In addition, immunofluorescent assay was conducted to corroborate the colocalization between NS and p27 in HCC cells. In this way, we revealed that NS showed apparent co-localization with p27 both in the nucleus and the nucleolus of HCC cells (Fig. 1D).

To further characterize the NS–p27 interaction, we constructed NS and p27 truncations (Fig. 2A and B) and mapped the binding domains of the two proteins. Wild type (wt) Flag-NS and its truncated mutants were co-transfected with myc-p27 in 293T cell, followed by immunoprecipitation assay. As shown in Fig. 2B, p27

![Fig. 1. NS interacts with p27 in cells. (A) Co-immunoprecipitation between Ectopically expressed NS and p27. 293T cells were co-transfected with Flag-NS and myc-p27. Cell lysates were immunoprecipitated with anti-myc antibody, and then subjected to western blot analysis. (B) Endogenous NS interacts with p27 in HCC cells. Lysates of HepG2 cells were immunoprecipitated with anti-p27, anti-NS antibodies, or control IgG. The immunocomplexes were subjected to western blot analysis using anti-NS and anti-p27 antibodies. (C) GST Pull-down analysis of p27 and NS interaction. 293T cells were transfected with Flag-NS and subjected to GST pull-down using GST or GST-p27 fusion protein. (D) Immunofluorescent analysis of the colocalization between NS and p27 in HCC cells. HepG2 and Hep3B cells were transfected Flag-NS and subjected to immunofluorescent assay using mouse monoclonal anti-Flag and rabbit polyclonal anti-p27 antibodies. Next, the slides were stained with goat anti-rabbit secondary antibody (red) and goat anti-mouse secondary antibody (green). Nuclei were stained with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)]
interacted with 118–228 aa region of NS but not with other truncated mutants (Fig. 2C). Similarly, we revealed that NS interacted with 94–198 aa region of p27 (Fig. 2D). These observations suggest that the interaction between NS and p27 may potentially influence p27 function.

NS facilitates nucleolar p27 ubiquitylation and degradation

Polyubiquitylation and subsequent degradation play a critical role in regulating the level of p27 during cell cycle progression. Recent investigations highlighted the importance of nucleolar proteins in mediating the ubiquitylation and proteolysis of several important tumor suppressors, including p53 and p19Arf [25]. We therefore speculated that the interaction between nucleolar NS and p27 may potentially influence p27 ubiquitylation and degradation. To determine whether NS facilitated p27 ubiquitylation and degradation, HepG2 cells were transfected with HA-Ub and treated with 20 μM proteasome inhibitor MG132 for 6 h. As shown in Fig. 3A, knockdown of endogenous NS inhibited p27 polyubiquitylation and maintained p27 stability. To further determine in which compartment NS modulated p27 ubiquitylation, we performed cell fractionation assay and isolated nucleoplasmic and nucleolar fractions. As shown in Fig. 3B, following MG132 treatment, the level of ubiquitylated p27 in whole cell lysate (Wc) was significantly enhanced. However, we found that Mg132 treatment did not increase the ubiquitylation of nucleoplasmic p27 (Np), while nucleolar fraction of p27 (No) displayed markedly polyubiquitylation in response to MG132 treatment (Fig. 3B). This piece of data indicates that nucleolar compartment of p27 is rapidly ubiquitylated in HCC cells. Moreover, we analyzed whether depletion of NS could alter the polyubiquitlyation of nucleolar p27. In the nucleolar fractions, silencing NS expression significantly reduced the ubiquitlated forms of p27 (Fig. 3C). These results suggest that NS facilitates the ubiquitylation of p27 in the nucleoli of HCC cells.

Fig. 2. Mapping the domains responsible for NS–p27 interaction. (A) Schematic diagram of full-length NS and its truncated mutants. (B) p27 binds to the NS118–228aa truncation. 293T cells were co-transfected with Flag-tagged full-length NS (wt) or its truncated mutants, and myc-p27. Cell lysates were immunoprecipitated with anti-Flag antibody, and then subjected to western blot analysis using the indicated antibodies. (C) Schematic diagram of full-length p27 and its truncated mutants. (D) NS binds to the p27△1–93aa truncation in cells. 293T cells were transfected with myc-tagged full-length p27 (wt) or its deletion mutants, together with Flag-NS. Cell lysates were immunoprecipitated with anti-myc antibody, and subjected to western blot analysis using the indicated antibodies.

Fig. 3. Depletion of NS impairs nucleolar polyubiquitylation of p27. (A) Knockdown of NS inhibits p27 polyubiquitylation in HepG2 cells. HepG2 cells were transfected with HA-Ub and control-shRNA or NS-shRNA (shNS#1 and shNS#2) constructs, and treated with MG132 for 6 h before harvest. These cell lysates were immunoprecipitated with anti-p27 antibody and subjected to western blot analysis with the indicated antibodies. (B) Ubiquitylated p27 are enriched in the nucleolar compartment. HepG2 cells were transfected with HA-Ub and treated with or without MG132 for 6 h before harvest. Then, biochemical fractionation was performed to acquire nucleoplasmic and nucleolar fractions. These subcellular fractions were immunoprecipitated using anti-p27 antibody and subjected to western blot analysis using the indicated antibodies. Wc, whole-cell lysate; Np, nucleoplasmic fraction; No, nucleolar fraction. (C) Knockdown of NS inhibits nucleolar ubiquitylation of p27. HepG2 cells were transfected with HA-Ub and NS-shRNA or control-shRNA (shNS#1 and shNS#2) plasmids, and treated with MG132 for 6 h before harvest, followed by biochemical fractionation. Then, acquired nucleolar fractions were used for ubiquitylation analysis of p27.
NS dissociates the interaction of p27 and CDK2

Ubiquitin-dependent degradation of p27 takes place in a CDK2–cyclin E-dependent process [26]. In addition, the dynamic association between p27 and CDK2 plays a key role in cell cycle regulation [27]. Then, we tested whether NS-mediated p27 ubiquitylation could disturb the interaction between p27 and CDK2. We discovered that ablation of NS not only enhanced the interaction between p27 and CDK2 but also induced the downregulation of CDK2 in HepG2 and Hep3B cells (Fig. 4A and B). At the same time, we found that knockdown of endogenous NS caused the reduction of cellular level of cyclin E and CDK2 in HCC cells (Fig. 4B). These findings implicate that NS-mediated p27 polyubiquitylation may influence the activity of CDK2.

Fig. 4. Knockdown of NS inhibited CDK2 activity. (A) NS dissociates p27–CDK2 interaction in HCC cells. HepG2 and Hep3B cells were transfected with control shRNA or NS-shRNA (shNS#1 and shNS#2) plasmids for 48 h. Cell lysates were immunoprecipitated with anti-CDK2 antibody, and subjected to western blot analysis. (B) Knockdown of NS inhibits the protein expression of CDK2 and cyclin E. HepG2 and Hep3B cells were transfected with NS-shRNA or control-shRNA plasmids for 48 h. These cell lysates were subjected to western blot analysis with indicated antibodies. The bar chart demonstrated the ratio of NS, p27, CDK2, cyclin E expression to GAPDH for the above by densitometry. The data are mean ± SEM (*, #, ‰, p < 0.05).
Knockdown of NS inhibits cell proliferation in HCC cells

It has been well documented that the interaction of p27 and CDK2 plays a key role in the control of CDK2 activation and cell proliferation [28]. Based on the above results that NS competed with CDK2 for p27 binding and led to p27 ubiquitylation and degradation, we speculated that NS might play a role in HCC cell cycle regulation and proliferation. To validate the hypothesis, NS-targeting shRNA plasmids were transfected into HCC cells, followed by EdU proliferation assay. As shown in Fig. 5A, knockdown

![Image of knockdown of NS inhibits cell proliferation in HCC cells]

Fig. 5. Knockdown of NS inhibits the proliferation of HCC cells. (A) Knockdown of NS downregulates the viability of HCC cells. HepG2 and Hep3B cells were plated into 96-wells and transfected with NS-shRNA (shNS#1 and shNS#2) or control shRNA plasmids. 48 h after transfection, the cells were subjected to EdU incorporation assay. The nucleus was stained with Hoechst 33342 (blue). Column graph shows the percentage of EdU-positive cells relative to Hoechst 33342 stained nuclei. The data are mean ± SEM (*p < 0.05). Scale bar, 100 μm. (B) Knockdown of NS reduces the cell growth rate of HCC cells. HepG2 and Hep3B cells were plated into 96-wells and transfected with NS-shRNA or control shRNA plasmids, followed by CCK-8 assay. The data are mean ± SEM (*p < 0.05). (C) Knockdown of NS induces G1 arrest. 48 h after transfection, HepG2 and Hep3B cells were stained with PI for DNA content analysis by FACS. The data are mean ± SEM (*p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
of NS remarkably reduced the proportion of EdU-positive cells, suggesting that knockdown of NS suppressed HCC cell proliferation. In addition, CCK-8 assay confirmed that HepG2 and Hep3B cells exhibited a dramatic decrease in cell proliferation following NS depletion (Fig. 5B). Furthermore, flow cytometry analysis revealed that depletion of NS drastically induced cell cycle arrest in the G1 phase (Fig. 5C). The data suggested that depletion of NS obviously resulted in proliferative impairment and G1 arrest in HCC cells.

Silencing NS expression reduced in vitro colony formation and in vivo tumor growth

We have demonstrated that NS promoted nucleolar ubiquitylation of p27 and enhanced the proliferation of HCC cells. Next, we determined whether NS could influence colony formation and in vivo tumorigenicity of HCC cells. HepG2 and Hep3B cells were infected with control shRNA or NS shRNA-expressing lentiviruses. Western blotting analysis verified that the expression of NS were significantly decreased following the infection of NS shRNA lentiviruses (Fig. 6A). As shown in Fig. 6B, depletion of NS markedly inhibited the colony formation capacities of HepG2 and Hep3B cells. Furthermore, the cells were subcutaneously injected into BALB/c nude mice with a number of \(5 \times 10^6\) cells per mouse. The tumor sizes were measured weekly. In this way, we found that NS-depleted tumors exhibited markedly impaired growth curve, compared with the control group (Fig. 6C and D). Likewise, the final tumor weights of NS-shRNA groups decreased obviously (Fig. 6E). The results indicated that knockdown of NS significantly suppressed HCC tumor growth in vivo.

The correlation between NS and p27 expression in HCC specimens

We further analyzed the prognostic significance of NS expression in patients with HCC. As shown in Fig. 7A and B, we found that NS expression was elevated in HCC tissues relative to the adjacent normal ones, which was inversely correlated with that of p27. Immunohistochemical staining was performed to determine the expression profiles of NS and p27 in HCC specimens. As expected, NS was highly expressed in HCC tissues, whereas p27 expression was remarkably downregulated in tumors tissues, compared with non-tumorous adjacent specimens (Fig. 7C). Linear regression analysis confirmed that there was a negative correlation between NS and p27 expression (Fig. 7D). Furthermore, statistical analysis indicated that NS expression was significantly associated with tumor differentiation \((p = 0.001)\), tumor metastasis \((p = 0.039)\) and AFP \((p = 0.020)\) in HCC (Table 1).

Finally, Kaplan–Meier analysis was performed to examine the correlation among NS and p27 expression and HCC patients’ survival. The results revealed that patients with high NS expression had significantly worse prognosis compared with that of patients with low NS levels (Fig. 8A). In contrast, high p27 expression could herald a better outcome in HCC patients (Fig. 8B). Finally, when both NS and p27 expression were taken into account to evaluate HCC prognosis, we reached the conclusion that low NS plus high p27 expression could predict longer cancer-free survival time (Fig. 8C).

Fig. 6. Knockdown of NS inhibits colony formation and in vivo tumorigenicity of HCC cells. (A) Western blot detection of lentiviral infection efficiency in HepG2 and Hep3B cells. HepG2 and Hep3B cells were infected with Control, shNS#1 (V1) or shNS#2 (V2) lentiviruses, 48 h after infection, lentiviral infection efficiency were determined using western blot analysis. The bar chart demonstrated the ratio of NS protein to GAPDH by densitometry. The data are mean \(\pm\) SEM \((^*p < 0.05)\). (B) Depletion of NS inhibits colony formation of HCC cells. Equal numbers of HepG2 and Hep3B cells infected with control, shNS#1 (V1) or shNS#2 (V2) lentiviruses were plated into 6-well plates. These cells were fixed and stained with crystal violet after incubated for 15 days. The cell colonies (>0.5 mm in diameter) were counted (mean \(\pm\) SEM, \(^*p < 0.05\)). (C) Knockdown of NS inhibits tumorigenic properties of HCC cells in vivo. Control, shNS#1 (V1) or shNS#2 (V2); HepG2 cells were injected subcutaneously into BALB/c nude mice. The left panel showed the mice bearing tumors from the control group; the middle panel showed the mice bearing tumors from the LV-NS-shRNA#1 (V1) group; the right panel showed the mice bearing tumors from LV-NS-shRNA#2 (V2) group. (D) Knockdown of NS impaired the tumor growth in vivo. Following cancer cell injection, the tumor volume were measured and calculated every 5 days \((n = 5)\) (mean \(\pm\) SEM, \(^*p < 0.05\)). (E) Knockdown of NS reduced mean final tumor weights. The final tumors were taken out and weighted on an electronic balance (mean \(\pm\) SEM, \(^*p < 0.05\)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Aberrant cell cycle regulation plays an important role in tumor development [29]. Serving as a key CDKI, p27 is closely related to HCC progression [30]. In the present study, we found that NS interacted with p27 and induced nucleolar polyubiquitylation of p27, leading to resultant degradation of p27 protein in HCC cells. Therefore, our data provide a new viewpoint on the regulation of p27 in hepatocarcinogenesis.

The nuclear localization of p27 is a prerequisite for it to function as a cell cycle regulator, while aberrant function of p27 in human tumors are partly attributed to nuclear export and degradation of p27. For example, Akt and S6K may mediate the phosphorylation and nuclear export of p27 to promote breast tumorigenesis [31,32]. Interestingly, some previous studies revealed that p27 might exhibit nucleolar distribution in malignant and somatic cells. An early study by Sgambato A et al. showed that p27 displayed a clear nucleolar signal in a proportion of bladder cancer specimens [33]. Immuno fluorescent analysis revealed that p27 co-localized with its binding protein Jab1 in the nucleolus of lung cancer cells [34]. Nucleolar-like distribution of p27 was also observed in some other studies [35,36]. Notably, a recent report found that C. elegans homologue of p27kip1, was distributed primarily in the nucleoli of hypodermal cells [37]. Moreover, nucleolar localization of p27 in these cells was regulated by β integrin signaling and p27 E3 ligase SKP2. These findings suggest that p27 nucleolar distribution may exist in a broad range of cells and involves complicated molecular mechanisms. However, the physiological significance of p27 nucleolar distribution remains unclear. We discovered apparent nucleolar distribution of p27 in HCC cells (Supplementary Fig. S1, Fig. 1D) and further revealed that nucleolar protein NS facilitated nucleolar ubiquitylation of p27 through direct interaction. In keeping with these observations, knockdown of NS alleviates NS-induced p27 nucleolar ubiquitylation and resultant degradation.

### Discussion

Fig. 7. The correlation between NS and p27 expression in HCC tissues. (A) The protein expression of NS was inversely correlated with that of p27. Eight pairs of HCC tissues and their adjacent normal tissues were subjected to western blot analysis using the indicated antibodies. PCNA was used as a marker protein of HCC samples and GAPDH was used as an internal control for protein load and integrity. (B) The bar chart demonstrated the ratio of NS and p27 expression to GAPDH between tumorous and non-tumorous tissues by densitometry. *p < 0.05. The data are mean ± SEM (p < 0.05, compared with the adjacent tumor tissues). (C) Representative images of NS and p27 immunohistochemical staining in HCC tissues and adjacent normal tissues. Scale bar, 100 μm. (D) Linear regression analysis of the correlation between NS and p27 expression in 118 HCC samples.

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<td>50</td>
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Statistical analyses were carried out using Pearson χ² test. *p < 0.05 was considered significant.
Therefore, our findings implicate that nucleolar distribution of p27 may facilitate its ubiquitylation and degradation, and apparent nucleolar distribution is potentially an indication of rapid p27 turnover in cancer cells.

The interaction between p27 and CDK2 regulates cells from G1 to S phase and maintains normal cell cycle progression [38]. The disruption of p27/CDK2 complex partly accounts for abnormal cell cycle and HCC development. However, the factors which give rise to the insulation of p27/CDK2 complex remain largely elusive. Though nuclear export of p27 has been widely regarded as an important mechanism underlying the insulation of p27/CDK2 interaction, cytoplasmic distribution of p27 was barely observed in HCC tissues and cell lines [30,39]. In the present study, we illustrated that NS bound to p27 in the nucleoli to dissociate p27/CDK2 interaction. Therefore, NS may compete with CDK2 for p27 binding, subsequently resulting in CDK2 activation and cell cycle progression.

Since the initial identification of NS in 2002 by Tsai et al., various studies have suggested that NS play a key role in multiple human tumors [40–42]. NS protein has a significantly higher expression in non-small cell lung cancer tissues than in normal lung samples [40]. Likewise, NS is highly expressed in esophageal tissues and knockdown of NS inhibits cell proliferation, caused G1/G0 phase arrest in Eca109 cells [42]. Moreover, increased expression of NS has been reportedly associated with HCC development [22]. Similarly, we discovered that NS was highly expressed in HCC tissues and cell lines (Fig. 7A, Supplementary Fig. S2) and depletion of NS suppressed HCC cells proliferation (Fig. 5). It was initially supposed that p53-mdm2 pathway played an important part in the tumor-promoting role of NS [43]. However, a recent study showed that NS remains indispensable in p53-null cancer cells, suggesting that NS may regulate cancer cell proliferation through p53-independent mechanisms [44]. In keeping with these findings, we showed that NS could regulate the proliferations of p53-wt (HepG2) and p53-null (Hep3B) liver cancer cells. Moreover, we delineated an involvement of NS in the regulation of p27 and CDK2 activities during liver tumorigenesis. These findings indicate that the regulation of p27 may serve as a novel and p53-independent mechanism underlying NS-mediated malignant proliferation.

In summary, we identified NS as a novel binding partner of p27 in HCC cells. NS promotes the polyubiquitylation of nucleolar p27 and alleviates its activity by dissociating CDK2–p27 interaction. Importantly, high levels of NS and low levels of p27 predict worsened outcome in patients with HCC. These findings expand our understanding of p27 in regulating cell cycle progression of HCC and provide new opportunities to develop molecular-targeted therapies in HCC.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.12.008.

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X. Huang, X. Qian, C. Cheng, S. He, L. Sun, Q. Ke, et al., Expression of PIRh2, a p27(Kip1) ubiquitin ligase, in hepatocellular carcinoma: correlation with p27(Kip1) and cell proliferation, Hum. Pathol. 42 (2011) 507–515.


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