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Original Article

Nucleostemin/GNL3 promotes nucleolar polyubiquitylation of p27^{kip1} to drive hepatocellular carcinoma progression

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ABSTRACT

p27^{kip}, 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.

p27^{kip}, 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

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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 MIF4GD 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 nucleolar 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 by morphologic and STR analyses, and the absence of mycoplasma contamination was also examined regularly. They were latest evaluated in March 14, 2016. The cells were maintained in DMEM (Sigma, St. Louis, MO) medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37 °C in 5% CO_2. Antibodies against NS (SC-67013), p27 (SC-528), ubiquitin (SC-8017), CDK2 (SC-748), cyclin E (SC-481) and PCNA (SC-56) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies against Flag (M2; F3165) and GAPDH (G9545) were purchased from Sigma. Antibodies against Myc (#2276) and Fibrillarin (#2639) were purchased from Cell Signaling Technology, Danvers, MA.

RNA interference

Control shRNA or NS-targeting shRNA vectors were constructed by inserting control shRNA or NS shRNA oligos into a hU6-MCS-Ubiquitin-EGFP-IRES-puromycin lentiviral vector (Genechem, Shanghai, China). The shRNA plasmids were subjected to HCC cell transfection using SuperFectin transfection reagent (Superfetin Biocompany, Shanghai, China) according to the manufacturer's instructions. 48 h after transfection, the cells were harvested and used for subsequent experiments. The target sequence of NS shRNA oligos were shRNA#1, 5′-AAG AAC TAA AAC AGC AGC AGA-3′; siRNA#2, 5′-CCT GAT ATT AAG CCA TCA AAT-3′. The sequence of control shRNA was 5′-TTC TCC GAA CGT GTC ACG T-3'. These shRNA constructs were subsequently subjected to lentiviral package by Genechem.

Subcellular fractionation

Cell fractionation to isolate nucleoli was performed as a previous report [23]. Briefly, 5×10^6 cells were harvested by trypsinization, washed three times with ice-colded PBS, resuspended in 1.5 ml Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 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 $MgCl_2$) and centrifuge 5 min at 1430 g. Then, the pellets were collected and resuspended with 0.5 ml S2 solution (0.35 M Sucrose, 0.5 mM $MgCl_2$). The samples were sonicated for 8×10 s 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 $MgCl_2$) 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 centrifugated for 5 min at 1430 g to acquire highly purified nucleoli.

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 × protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and centrifuged at 4 °C, 13,000 g for 15 min. After collecting the supernatant, the protein concentration was determined using a BCA protein assay kit (Bio-Rad, Hercules, CA). Then, the samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidine diflouride filter (PVDF) membranes. The membranes were blocked with 5% dry milk in TBST for 2 h and incubated with primary antibodies overnight at 4 °C. After washing three times, the members were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h. Finally, the bands were acquired by the enhanced chemiluminescence (ECL) detection system. Alternatively, secondary antibody incubation was conducted using IRDye® secondary antibodies (Odyssey LI-COR, Lincoln, NE, USA), and the protein bands were visualized using Odyssey infrared imaging system.

For immunoprecipitation, cell lysates were incubated with 6 μg of primary antibodies or control immunoglobulin G (IgG) overnight at 4 °C. Next, 35 μl Protein G-Sepharose (Sigma) was added for rocking 2 h at 4 °C. Then, the precipitates were washed three times, boiled for 5 min with SDS sample buffer and subjected to western blot analysis.

GST pull-down assay

GST and GST–p27 fusion proteins were induced with 0.1 mM IPTG in Rosetta (DE3) bacteria for 10 h at 37 °C, and then purified using Glutathione-Sepharose 4B beads. The Flag-tagged NS plasmid was transfected into HEK293T cells and the cell lysate was incubated with purified Sepharose bead-bounded GST or GST-p27 protein for 30 min at 4 °C. After washing three times, the bead samples were analyzed by western blotting with an anti-Flag antibody (M2; Sigma) or subjected to coomassie blue staining.

Immunofluorescent analysis

Forty-eight hours after transfection, the cells were fixed with 4% formaldehyde in PBS for 1 h and permeabilized with 1% Triton X-100 for 10 min at room temperature. Next, the cells were blocked with 1% BSA for 2 h and incubated with primary antibodies overnight at 4 °C. After washing three times with PBS, the secondary antibodies (Alexa Fluor 568- conjugated goat anti-rabbit or Alexa Fluor 488-conjugated goat anti-mouse IgG; Invitrogen, Carlsbad, CA) were incubated for 2 h at room temperature in the dark. Meanwhile, 10 μg/ml DAPI was added to stain the nucleus. Lastly, the slides were mounted and visualized using a Nikon confocal microscope (Nikon, NY, USA).

In vivo ubiquitylation assay

HCC cells were transfected with HA-Ub and control-shRNA or NS-shRNA plasmids for 48 h, and treated with 20 μM MG132 (Sigma) for 6 h. Then, the protein samples were immunoprecipitated using anti-p27 antibody, followed by western blot analysis using anti-Ub antibody.

Cell proliferation assay and colony formation assay

5-ethynyl-2′-deoxyuridine (EdU) incorporation and Cell Counting Kit-8 (CCK-8) assays were employed to assess the proliferation of HCC cells. EdU incorporation assay was performed using a commercial kit (EdU; Ribobio, China) and CCK-8 assay was conducted using a commercial kit (Dojindo, Kumamoto, Japan) in accordance