Original Article
NET-1 promotes the proliferation of hepatocellular carcinoma cells via activating P13K/Akt and JAK2/STAT3 signaling pathways

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Abstract: Objective: This study aimed to investigate the association of neuroepithelial transforming gene-1 (NET-1) between its related signaling pathways in hepatocellular carcinoma (HCC) pathogenesis. Methods: HCC cells with relative higher NET-1 expression were transfected with NET-1 siRNA. Cell proliferation, apoptosis, and cell cycle were detected. Expression or activation of related signaling pathways was also detected. Results: NET-1 showed the highest expression level in SMMC 7721 cells and siRNA transfection downregulated NET-1 mRNA expression compared with control cells. Moreover, cells transfected with NET-1 siRNA decreased in cell proliferation by arresting cell cycle at G1 phase, decreased in expression of activated status factors of P13K/Akt and JAK2/STAT3 signaling pathways, Bcl-2 and cyclin D1. Whereas, NET-1 siRNA promoted SMMC 7721 cells apoptosis. Conclusions: Downexpression of NET-1 promoted cell apoptosis and inhibited cell proliferation via cyclin D1 mediated inactivation of JAK2/STAT3/ Bcl-2 and P13K/Akt/Bcl-2 signaling pathways in HCC cells. Inhibition of NET-1 might be explored as a potential therapeutic target for HCC.

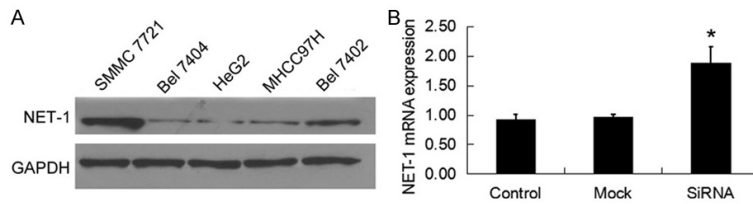**Keywords:** Hepatocellular carcinoma, NET-1, cell proliferation, SMMC 7721

Introduction

Hepatocellular carcinoma (HCC) is a cancer with high incidence and lethality [1]. HCC mainly resulted from chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections [2, 3]. Therapies for HCC are long-term and pressing tasks with poor results owing to the complex processes of HCC pathogenesis and metastasis, which involve in dysregulation of multiple signaling pathways and abnormal expression of related factors. Epithelial mesenchymal transition (EMT) in HCC is one of the major signs [4]. As reported, EMT in human cancers associates with dysregulation of multiple signaling pathways such as TGF-β signaling [5], phosphatidylinositol-3-kinase (P13K)/Akt signaling pathway [6], and janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway [7]. Moreover, neuroepithelial transforming gene-1 (NET-1) was reported to contribute to HCC cells proliferation and angiogenesis [8, 9]. Dual inhibition of NET-1 with vascular endothelial growth factor (VEGF) or sur-

vivin had been reported to inhibit cell growth and angiogenesis in HCC or skin squamous cell carcinoma, respectively [9, 10]. Previous studies had determined the upregulation of NET-1 expression level in HCC tissues than in peritumor or paracarcinoma tissues, with an positive association to tumor grading and clinical stages [11, 12]. Additionally, STAT3 is constitutively activated in HCC tissues [13] and upregulation of P13K is detected in HCC tissues [14]. However, there were no studies focusing on the interactions between NET-1 expression and P13K/Akt or JAK2/STAT3 signaling pathways in HCC pathogenesis.

To investigate the association of NET-1 between its related signaling pathways in HCC pathogenesis, we knocked down the expression of NET-1 in HCC cells and detected the biological behavior. Activation of EMT associated signaling pathways including P13K/Akt or JAK2/STAT3 signaling pathways were determined. This study would provide us with novel information focusing on association of NET-1 mediated HCC prolifer-

Figure 1. NET-1 expression in human hepatocellular carcinoma (HCC) cell lines. A. NET-1 protein expression in HCC cell lines were detected using Western blot analysis. B. NET-1 mRNA relative expression level in transfected cells were detected using qRT-PCR.

ation with P13K/Akt or JAK2/STAT3 signaling pathways, and the potential therapy for HCC by targeting NET-1.

Materials and methods

Cell lines and cell culture conditions

Human HCC cell lines including SMMC 7721, BEL 7404, HepG2, MHCC97H, and Bel-7402 (Chinese Academy of Sciences, Beijing, China) were incubated in DMEM supplemented with 10% FBS (Hyclone, Thermo Scientific, Epsom, UK) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C 5% CO_2.

Cell transfection

A total of 1×10^5 cells were seeded in each well of 24-well plates. SiRNAs with sequence targeting NET-1 (sense: 5'-CCACAAUGGCUGAGC-ACUUdTdT-3' and antisense: 3'-TdTdGGUGUU-ACCGUCACGUGAA-5') were synthesized by GenePharma (Shanghai, China). SiRNA against NET-1 were transfected into HCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufactures' instruction. Cells transfected with siRNA-NET-1-NC (Mock transfection) were served as negative control. Cells were cultured at 37°C 5% CO_2 after transfection.

Cell proliferation assay

Cell proliferation of transfected HCC cells was assessed using Cell Counting Kit 8 (CCK8, Beyotime, Shanghai, China) assay as described [15]. Briefly, transfected HCC cells were trypsinized at 0, 12, 24, and 48 h post transfection. Cell suspension were transferred to 96-well plates and incubated for 24 h. Then, cells were additionally incubated with CCK8 solution for 2

h, followed by a microplate spectrophotometer (Bio-Rad Labs, Sunnyvale, CA) analysis for the optical density of cells at 450 nm absorbance (A450). Each experiment was performed in triplicates.

Cell cycle analysis

Cell cycle analysis of transfected HCC cells was performed using flow cytometry analysis after 48 h transfection [16]. Cells were harvested by trypsin (Gibco Laboratories, Grand Island, NY, USA) and fixed with ethanol for 24 h. Then cells were stained with propidium iodide (PI, Biyuntian Biological Technology, Shanghai, China) and analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Each experiment was performed in triplicates.
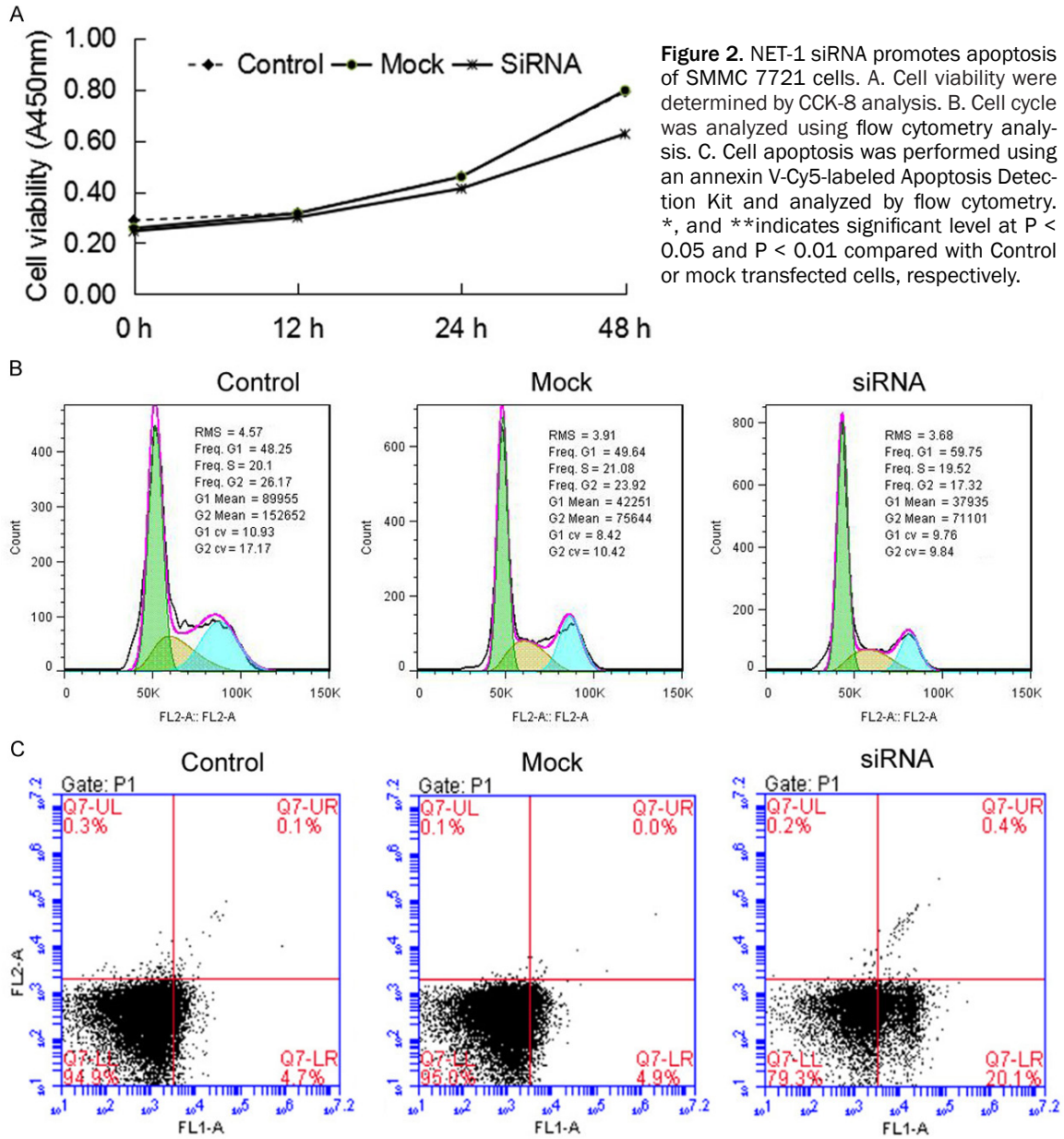
Cell apoptosis assay

Cell apoptosis assay of transfected HCC cells was performed using an annexin V-Cy5-labeled Apoptosis Detection Kit (Beyotime Institute of Biotechnology) and analyzed by flow cytometry [17]. Briefly, cells were harvested, washed, and pelleted after transfection for 24 h. Subsequently, cells were resuspended by 5 μL Annexin V-binding buffer containing Annexin V-Cy5 and 5 μL PI for 10 min, followed by analysis using a FACS Calibur flow cytometer (BD Biosciences). Percentages of annexin V-Cy5 positive and PI-negative cells (Annexin V+/PI-, early apoptotic cells) were calculated.

Western blotting analysis

Western blotting was performed as previously described. Cells were lysed and protein concentrations were measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Cell lysates were separated in 10% SDS-PAGE and transferred onto Millipore polyvinylidene fluoride membranes (PVDF, Millipore, Billerica, MA, USA). Membranes were then blocked in PBST (0.1% triton in PBS) and incubated with primary antibodies against NET-1 (1:200 dilution, Santa Cruz Biotechnology, Inc., CA, USA), cyclin D1 (1:1000, dilution, Cell Signaling Technology, CST, Denvers, MA, USA), Janus

Figure 2. NET-1 siRNA promotes apoptosis of SMMC 7721 cells. A. Cell viability were determined by CCK-8 analysis. B. Cell cycle was analyzed using flow cytometry analysis. C. Cell apoptosis was performed using an annexin V-Cy5-labeled Apoptosis Detection Kit and analyzed by flow cytometry. *, and **indicates significant level at P < 0.05 and P < 0.01 compared with Control or mock transfected cells, respectively.

kinase 2 (JAK2, 1:200 dilution, CST), phosphorylated (p)-JAK2 (1:200 dilution, CST), STAT3 (1:1000 dilution, Abcam, Cambridge, UK), p-STAT3 (1:1000 dilution, Abcam), Bcl-2 (1:1000 dilution, Santa Cruz Biotechnology), Bax (1:1000 dilution, Abcam), and GAPDH (1:1500 dilution, CST) at 4°C overnight, followed by incubation with secondary antibodies for 1 h. The polypeptide bands were detected using an ECL detection system (Pierce, Rockford, IL, USA) and quantified with AlphaEase software (Alpha, USA).

Immunocytochemical analysis

Immunocytochemistry were performed on the fixed cells to detect p-Akt and p-P13K expression. Slides were dewaxed, blocked with normal goat serum and sequentially incubated with specific primary antibodies against p-Akt (1:200 dilution, CST) and p-P13K (1:400 dilution, CST) at 4°C overnight and biotinylated secondary antibody at 37°C for 4 h. Signal visualization was performed with diaminobenzidine (DAB) staining. Histological scoring was evalu-
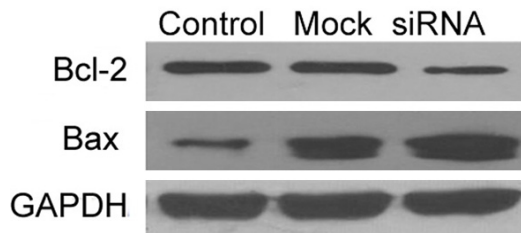
Figure 3. NET-1 siRNA upregulated Bax/Bcl-2 ratio in SMMC 7721 cells. Proteins expression in SMMC 7721 cells were detected using Western blot analysis.

ated by a double blind scoring method. Cells with brown granules were considered as p-Akt and p-P13K positive cells. Positive cell percentages were calculated as: averaged positive cells numbers/total cell numbers of five random fields.

RNA isolation and quantitative real-time PCR

Total RNA from HCC cells was extracted using TRIzol Reagent (Invitrogen) 48 hours after transfections. Cell lysates were treated with RNase-free Dnase I (Promega Biotech, USA), and first-strand cDNA was then synthesized using a Reverse Transcriptase Kit (TaKaRa, China). Expression levels of NET-1 mRNA were determined by using SYBR ExScript qRT-PCR Kit (TaKaRa) on an Applied Biosystems (ABI) PCR System 9700 (ABI, Foster City, CA, USA). GAPDH were used as the internal control for NET-1 mRNA relative expression level. Primers used for targets amplification were synthetized by Shanghai Sangon Biological Engineering Technology and Services Ltd. (Shanghai, China). Primers sequences for NET-1 were 5'-GTGGCTTCACCAACTATACG-3' (forward) and 5'-GACTGCATTAGTTCGGATGT-3' (reverse), and primers sequences for GAPDH were 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). The reaction conditions were: 95°C 10 min, followed by 95°C 30 s, 60°C 40 s for 40 cycles. All reactions were run in triplicates, and relative expression level of NET-1 mRNA was analyzed using $2^{-\triangle\triangle Ct}$ method.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of triplicates. Statistical analysis was performed using SPSS 19.0 by a one-way analysis of variance (ANOVA) test for difference

analysis among groups. $P < 0.05$ was considered to be statistically significant.

Results

NET-1 shows relative higher expression level in SMMC 7721 cells

According results of Western blot analysis, NET expressed highest in SMMC 7721 cells compared with the others (**Figure 1A**). NET-1 protein showed relative lower expression levels in Bel-7404, HepG2, and MHCC97H cells. So we selected the SMMC 7721 cells and performed siRNA transfection on it (**Figure 1B**).

NET-1 siRNA inhibits cell proliferation and promotes cell apoptosis

SMMC 7721 cells were transfected with NET-1 siRNA and cell proliferation was assayed using CCK-8 methods. Results showed that knockdown of NET-1 in SMMC 7721 cells significantly inhibited cell viability by reducing A450 nm values ($P < 0.01$, **Figure 2A**). Cell cycle analysis showed that SMMC 7721 cells treated with NET-1 siRNA significantly arrested cell cycle at G1 phase (**Figure 2B**). Cell numbers in S and G2/M phases were significantly reduced by NET-1 siRNA transfection, in comparison with those of the Control and Mock transfected cells. Cell apoptosis analysis showed downexpression of NET-1 by siRNA significantly upregulated the percentages of Annexin V+/PI- early apoptotic cells (**Figure 2C**). All these data showed NET-1 contributed to cell proliferation, and NET-1 siRNA transfection obviously inhibited proliferation and promoted apoptosis of SMMC 7721 cells.
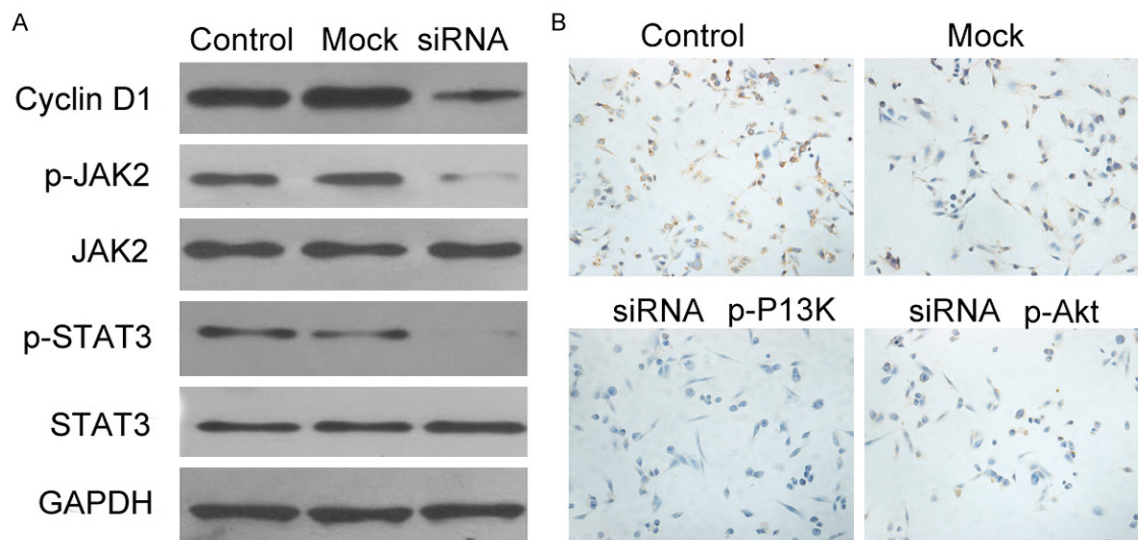
NET-1 siRNA upregulated Bax/Bcl-2 ratio

Since NET-1 siRNA inihibited cell proliferation and promoted cell apoptosis, we detected the expression of two apoptotic related proteins, Bcl-2 and Bax [18, 19]. Western blot analysis showed NET-1 siRNA significantly inhibited expression of Bcl-2 protein and promoted expression of Bax protein (**Figure 3**). That demonstrated NET-1 siRNA transfection into SMMC 7721 cells upregulated ratio of Bax/Bcl-2.

NET-1 siRNA inactivates JAK2/STAT3 signaling pathway

Both cyclin D1 and JAK2/STAT3 signaling pathway had been proved to promote cell prolifera-

Figure 4. NET siRNA inactivates JAK2/STAT3 and P13K/Akt signaling pathways. A. Expression of cyclin D1 and JAK2/STAT3 signaling pathway factors were detected by Western blot analysis. B. Expression of P13K/Akt signaling pathway factors were analyzed using immunocytochemistry.

tion and differentiation [20, 21]. Expression of cyclin D1 promotes cell proliferation via JAK2/STAT3 pathway [22, 23]. Western blot analysis showed expression of cyclin D1 was inhibited in NET-1 siRNA transfected SMMC 7721 cells (**Figure 4A**). Compared with control or mock transfected cells, NET-1 siRNA transfected showed downregulated expression of cyclin D1, p-JAK2 and p-STAT3 proteins. Parallel expression levels of cyclin D1, p-JAK2 and p-STAT3 proteins were observed in control and mock transfection cells, and parallel expression levels of JAK2 and STAT3 were observed in three groups. These results demonstrated that NET-1 siRNA inhibited cell proliferation via inactivating cyclin D1 mediated JAK2/STAT3 signaling pathway.

NET-1 siRNA inactivation of P13K/Akt signaling pathway

Immunocytochemistry were performed to detect expression of p-Akt and p-P13K proteins in NET-1 siRNA transfected SMMC 7721 cells. Immunocytochemical analysis results showed that p-Akt and p-P13K positive cells numbers (cells with brown granules) in NET-1 siRNA transfected cells were reduced in comparison with control and mock transfected cells (**Figure 4B**). These showed NET-1 siRNA inactivated P13K/Akt signaling pathway.

Discussion

EMT in HCC relates to activated P13K/Akt or JAK2/STAT3 signaling pathways and cell apoptosis and metastasis [4, 6, 7]. Moreover, NET-1 contributes to HCC cells proliferation and angiogenesis and inhibition of NET-1 might inhibit HCC cell growth and angiogenesis [8, 9]. However, there were no studies focusing on the interactions of NET-1 expression with P13K/Akt or JAK2/STAT3 signaling pathways in HCC. In this present study, we demonstrated that NET-1 contributed to HCC cells proliferation via activation of P13K/Akt or JAK2/STAT3 signaling pathways.

NET-1 overexpression had been reported in HCC tissues [11, 12, 24]. NET-1 aberrant expression positively associated with HCC metastasis and poor survival [8, 9], and inhibition of NET-1 in SMMC 7721 cells inhibited cell proliferation and cell cycle, as well as promoted cell metastasis and invasion suggesting NET-1 might be a therapeutic target for HCC [24]. In this present study, we confirmed and demonstrated that NET-1 siRNA transfected SMMC 7721 cells promoted cell apoptosis and cell cycle arrest in G1 phase, and conversely inhibited cell proliferation compared with control cells. These changes in biological behaviors showed NET-1 contributed cell proliferation and

inhibition of NET-1 might be a therapeutic target for HCC.

Constitutive expression or activation of Bcl-2 signaling, P13K/Akt and JAK2/STAT3 signaling pathways positively associates with EMT processes [6, 7, 25]. Reports had showed that activation of Bcl-2 signaling, P13K/Akt and JAK2/STAT3 signaling pathways promoted HCC cells proliferation and invasion [26-29]. Overexpression of cyclin D1 gene, Bcl-2, P13K/Akt and JAK2/STAT3 signaling pathways in aggressive HCC was determined in previous studies [30-32]. Moreover, expression of cyclin D1 had been proved to promote cell proliferation via JAK2/STAT3 and P13K/Akt signaling pathways [33-36]. In this present study, we revealed that expression of Bcl-2, p-JAK2, p-STAT3, p-P13K and p-Akt, as well as cell proliferation were inhibited by NET-1 siRNA transfection in SMMC 7721 cells. These results suggested that NET-1 siRNA inhibited HCC cells proliferation via cyclin D1 mediated JAK2/STAT3/Bcl-2 and P13K/Akt/Bcl-2 signaling pathways.

Conclusion

In conclusion, we confirmed that downexpression of NET-1 in HCC cells promoted cell apoptosis and inhibited cell proliferation via cyclin D1 mediated inactivation of JAK2/STAT3/Bcl-2 and P13K/Akt/Bcl-2 signaling pathways. However, more studies should be done to investigate expression of EMT factors and the association NET-1 between EMT pathogenesis in our future studies to explore the association between EMT and NET-1 expression and the mechanism behand it.

Disclosure of conflict of interest

None.

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