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NET-1 knockdown inhibits proliferation and promotes apoptosis of hepatocellular carcinoma cells by regulating apoptosis-related proteins and the PI3K/Akt signaling pathway.

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Abstract

Background: Neuroepithelial transforming gene-1 (*NET-1*) is a guanine nucleotide exchange factor that activates Rho family proteins. We aimed to evaluate the effect of siRNA-mediated knockdown of *NET-1* on proliferation and apoptosis of hepatocellular carcinoma (HCC) cells and the underlying mechanisms.
Material and methods: *NET-1* mRNA and protein levels were detected in four HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell line L-02 using RT-PCR and western blot. Cell proliferation was evaluated by the CCK-8 assay, and apoptosis was assessed by flow cytometry. Protein levels of apoptosis-related proteins and PI3K/Akt pathway proteins were evaluated by western blot.
Results: *NET-1* levels were significantly higher in the four HCC cell lines than those in the normal liver cell line L-02; the highest levels were observed in MHCC97-H cells. Knockdown of *NET-1* by siRNA inhibited proliferation and promoted apoptosis of HCC cells. In addition, *NET-1* knockdown decreased *Bax* and *cyclin D1* expression, but increased *Bcl-2* and caspase-3 levels in HCC cells. The PI3K/Akt pathway was blocked by knockdown of *NET-1*.
Conclusion: *NET-1* knockdown inhibits proliferation and promotes apoptosis of HCC cells by regulating the expression of apoptosis-related proteins including *Bax*, *Bcl-2*, *cyclin D1*, and caspase-3, and the PI3K/Akt pathway.

Keywords: *NET-1*, Hepatocellular carcinoma, Apoptosis, Proliferation.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third leading cause of cancer-related death worldwide [1]. It is an increasingly important public health problem and its overall incidence remains alarmingly high in the developing world [2]. In the past two decades, despite extensive research and significant advancements in the diagnosis and treatment of HCC, the prognosis of the disease remains poor [3]. The process of HCC cell proliferation and apoptosis consists of a complex of series of sequential steps involving coordination of diverse signal transduction pathways [4,5]. A better understanding of the mechanism of proliferation and apoptosis of HCC will help to develop novel therapeutic strategies and improve patient survival.

Neuroepithelial transforming gene-1 (*NET-1*) is a member of the guanine nucleotide exchange factor (GEF) family that activates Rho family proteins. It was originally identified as an oncogene in neuroepithelial cells. Several recent studies showed dysregulation of *NET-1* expression in HCC. Shen et al. reported that the mRNA expression of *NET-1* was markedly up-regulated in human HCC tissues in comparison to matched paracarcinoma tissues, and *NET-1* expression was significantly higher in TNM III-IV HCC tissues than that of TNM I-II HCC tissues [6]. In addition, *in vitro* studies demonstrated that knockdown of *NET-1* expression by short interfering RNA (siRNA) significantly inhibited proliferation of HepG2 and SMMC-7721 HCC cells [7-9]. A recent study by Ye et al. showed that *NET-1* knockdown effectively decreased migration, invasion and metastasis of the HCC MHCC-97H cell line through interactions with merlin, the product of the neurofibromatosis type 2 gene [10]. Despite of these findings, our understanding of the relation of *NET-1* with HCC remains limited.

In this study, we aimed to evaluate the effect of siRNA-mediated knockdown of *NET-1* on proliferation and apoptosis of HCC cells and the underlying mechanisms. We assessed *NET-1* mRNA and protein levels in four HCC cell lines and

normal liver cells, and selected the HCC cell line with the highest level of *NET-1*. After transfection with *NET-1* siRNA and siNC, cell proliferation and apoptosis were evaluated. The expression levels of apoptosis-related proteins and the PI3K/Akt pathway were assessed to explore the molecular mechanism by which *NET-1* affects proliferation and apoptosis of HCC cells.

Material and Methods

Cell culture

All experimental procedures were approved by the medical ethics committee of Linyi People's Hospital. Human HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell line L-02 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37°C. Exponentially growing cells in culture were used for the experiments.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from cell lines using TRIzol reagent. 1 mL of Trizol was added, and the solution was mixed homogeneously for 10 min. The mixture was then transferred into eppendorf tubes (EP, 1.5 mL) with 200 μL chloroform. After shaking for 15 min, the EP tubes were centrifuged at 4°C for 15 min (12000 × g). The supernatant was transferred into other EP tubes and mixed with isopyknic isopropanol for 15 sec. Centrifugation (4°C, 10 min, 12000 × g) was carried out again, and the supernatant was discarded. The precipitate was washed by 75% ethonal twice and dissolved into 30 μL diethypyrocarbonate (DEPC) after drying to obtain RNA stock solution. The concentration of RNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and the RAN solution was stored at -80°C for further use. Genes were amplified by specific oligonucleotide primer, and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. Briefly, reaction conditions comprised of 55°C for 30 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Relative expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Cells were inoculated to 6-well plates (5 × 10^5 cells each plate) and cultivated for 24 h. The culture solution was absorbed away and cells were rinsed with cold phosphate-buffered saline (PBS) for 3 times. Cells were lysed using the radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of proteins were loaded on 12% sodium dodecyl sulfate polyacrylamide gel and separated by electrophoresis. Then, proteins were transferred onto nitrocellulose membranes (Hybond, Escondido, CA, USA). Membranes were blocked with Tris-buffered saline Tween-20 (TBST) containing 5% skimmed milk powder for 1 h at room temperature. After washing with TBST, membranes were incubated with primary antibodies diluted with 5% bovine serum albumin (BSA). Proteins were visualized using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit-Haemek, Israel).

Cell transfection

All transfection experiments were performed using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The *NET-1* siRNA were synthesized and modified chemically (Invitrogen, Shanghai, China). Cells were harvested for proliferation and apoptosis assays 72 h after transfection.

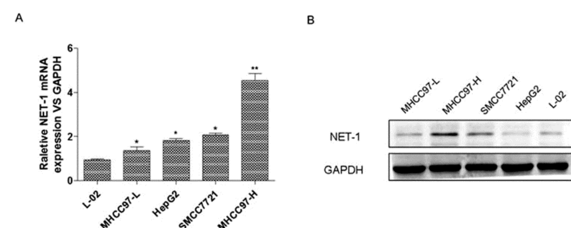

*Figure 1. A) The mRNA levels of NET-1 in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell lines L-02 by RT-PCR. *P<0.05 and **P<0.01, compared with L-02 cells, NET-1 mRNA expression in HCC cell lines had statistical difference. B) The protein expression of NET-1 in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell lines L-02 by western blotting.*

Flow cytometry (FCM)

Flow cytometric analysis was used to evaluate the influence of expression changes of *NET-1* on cell cycle progression and apoptosis in the human HCC cell line MHCC97-H. The cells in logarithmic-phase were selected and cultured in a 96-well plate at a density of 2 × 10^3 cells/well for 16 h in RPMI 1640 medium. After being subjected to treatment in triplicate wells, the cells were washed twice in PBS (2.68 mM KCl, 1.47 M KH_2PO_4, 8 mM Na_2KPO_4, 136.75 mM NaCl) and counted. A total of 50 to 100, 000 cells were collected and centrifuged for 5 min at 1000 rpm. 195 μL of Annexin V-FITC mixed liquor was added to resuspend cytotrophoblast cells. Then, 5 μL was added and mixed. Centrifugation (1000 rpm, 5 min) was performed after cultivation for 10 min. After discarding the supernatant, 10 μL of propidium iodide (PI) was added, and the sample was stilled in dark for 30 min. Finally, cell apoptosis was detected using FCM on the Moflo (Dako Cytomation, Glostrup, Denmark).

Cell proliferation assay

Cell proliferation was evaluated using a cell proliferation kit, cell counting kit-8 (CCK-8; Dojindo Molecular technologies,

Inc., Kyushu,Japan) according to the manufacturer's instructions. Cells were seeded into 96-well tissue culture plates at a density of 2 × 10^3 cells/well the day before transfection. Cell growth was analyzed at a wave length of 450 nm at 12, 24, 48 h after transfection using Envision (PerkinElmer). Experiments were performed in triplicate.

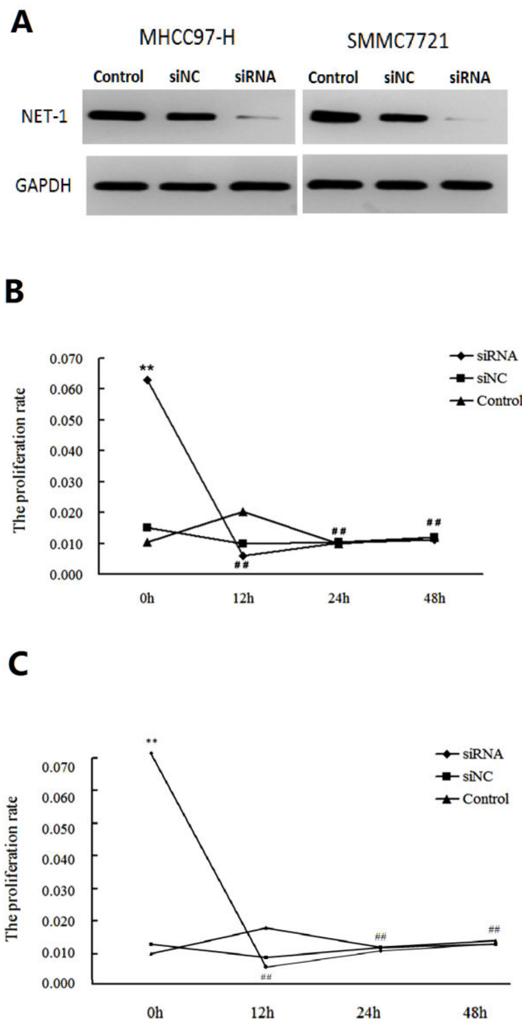

*Figure 2. A) The protein levels of NET-1 in HCC cell lines after transfection with NET-1 siRNA or siNC. B) The proliferation rate of MHCC97-H cells after transfection with NET-1 siRNA or siNC. C) The proliferation rate of SMMC7721 cells after transfection with NET-1 siRNA or siNC. **P<0.01, compared with the control group, proliferation rate of MHCC97-H cells in the siRNA group at 0 h had statistical difference. # #P<0.01, compared with 0 h, proliferation rate of MHCC97-H cells at 12, 24 and 48 h in the siRNA group had statistical difference.*

Statistical analysis

Statistical analysis was performed using SPSS version 19.0. All data were expressed as means ± SD from at least three independent experiments. P values were determined using one-way ANOVA. Statistical significance was defined as P<0.05.

Results

Screening of HCC cells with high expression of NET-1

We detected mRNA and protein levels of *NET-1* in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell line L-02 using RT-PCR and western blot. The results of RT-PCR showed that the level of *NET-1* in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721 and HepG2 was much higher than that in the normal liver cell line L-02. The highest *NET-1* level was found in MHCC97-H cells among these HCC cell lines (Figure 1A). The results of western blot were shown in Figure 1B. As shown, *NET-1* protein levels in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721 and HepG2 were higher than those of L-02 cells. The highest expression of *NET-1* was seen in MHCC97-H cells among these HCC cell lines. Therefore, MHCC97-H cells were selected for further study.

Knockdown of NET-1 inhibited the proliferation of HCC cells

The knockdown efficiency of *NET-1* in MHCC97-H cells was detected by Western Bloting (Figure 2A).The proliferation rate of MHCC97-H cells after transfection with *NET-1* siRNA was determined using the CCK-8 assay. The results showed that the proliferation rate of MHCC97-H cells in the siRNA treated group was much higher than that of the control group at 0 h, but it decreased significantly at 12, 24 and 48 h, after transfection (Figure 2B). Similar results were detected in SMMC7721 (Figure 2C).

Knockdown of NET-1 promoted HCC cell apoptosis

We assessed the percentage of apoptotic MHCC97-H cells and cell cycle by FCM. The results showed that the percentage of apoptotic MHCC97-H cells increased after transfection with *NET-1* siRNA compared with the control and siNC groups (Figure 3A). In addition, cell cycle analysis demonstrated that after transfection with *NET-1* siRNA, MHCC97-H cells were arrested in the G1/S phase (Figure 3B).

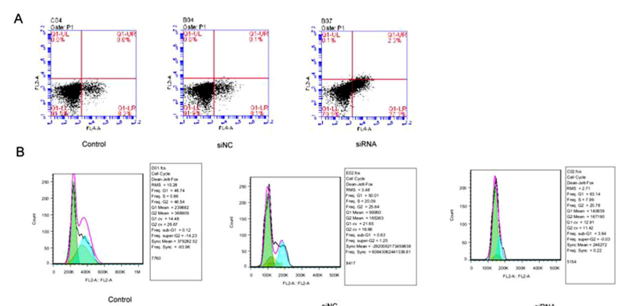

Figure 3. A) The percentage of apoptotic MHCC97-H cells in the control, siNC and siRNA groups. B) The cell cycle distribution of MHCC97-H cells in the control, siNC and siRNA groups.

Knockdown of NET-1 influenced the expression of apoptosis-related proteins and PI3K/Akt signaling pathway

To explore the molecular mechanism for the effect of *NET-1* on HCC, we assessed protein expression levels of *Bax*, *cyclin D1*, *Bcl-2* and caspase-3, finding that the levels of *Bax* and *cyclin D1* in MHCC97-H cells decreased significantly after transfection with siRNA, whereas the expression of *Bcl-2* and caspase-3 was up-regulated (Figure 4A). The effect of *NET-1* knockdown on the PI3K/Akt signaling pathway was also checked. Western blotting analysis showed that there was no change in the protein expression of PI3K, but the expression of Akt significantly decreased after transfection with *NET-1* siRNA (Figure 4B).

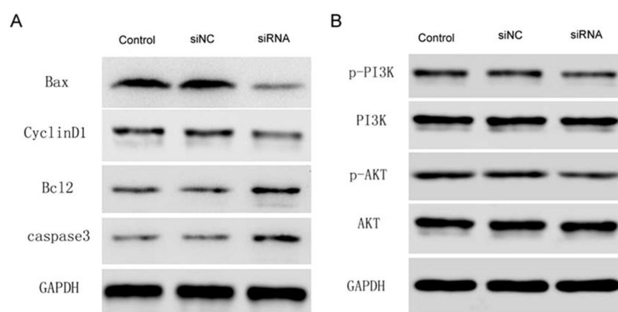

Figure 4. *A) The protein expression of Bax, cyclinD1, Bcl2 and caspase-3 by western blotting. B) The protein expression of p-PI3K, PI3K, p-AKT and AKT by western blotting.*

Discussion

HCC is the sixth most common cancer in the world and accounts for nearly 80% to 90% of all primary liver cancers [2]. The disease is more common in men than in women, and its incidence is increasing due to the dissemination of hepatitis B and C virus infection. The prognosis of HCC remains poor, with a five-year survival rate of approximately 10% [11]. Although there are many treatment options for HCC, there is still no cure for the disease. The identification of novel molecular targets for HCC is a challenge. In recent years, several studies have reported a link between *NET-1* and HCC. In this study, we aimed to evaluate the effects of *NET-1* knockdown on proliferation and apoptosis of HCC and the underlying molecular mechanisms. The main findings of our study are as follows: (a) the expression levels of *NET-1* were significantly higher in four HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2 than those in the normal liver cell line L-02; the highest levels were observed in MHCC97-H cells, (b) knockdown of *NET-1* by siRNA significantly inhibited proliferation and promoted apoptosis of HCC cells, and (c) *NET-1* knockdown affected the expression of apoptosis-related proteins and the PI3K/Akt pathway in HCC cells. These findings suggest that *NET-1* may be a potential therapeutic target for the treatment of HCC.

To elucidate the molecular mechanisms by which *NET-1* knockdown inhibited proliferation and promoted apoptosis, we examined the expressions of the key apoptosis regulators including *Bax*, *cyclin D1*, *Bcl-2* and caspase-3. Our results demonstrated that the expression levels of *Bax* and *cyclin D1* in MHCC97-H cells were significantly decreased after transfection with *NET-1* siRNAs, whereas the levels of *Bcl-2* and caspase-3 increased markedly. *Bax* is a pro-apoptotic member of the *Bcl-2* family of genes which regulate programmed cell death. The *Bax* protein shares highly conserved domains with *Bcl-2*, some of which are required for the formation of *Bax–Bcl-2* heterodimers [12]. Dysfunction of the p53/*Bax*/caspase-3 apoptosis signaling pathway has been shown to play a role in tumorigenesis and tumor progression [13]. A functional imbalance between *Bax* and *Bcl-2* is relevant for cancer cell behavior, cell invasion, cell adhesion, and metastatic potential [14]. *Cyclin D1* is a proto-oncogene that is overexpressed in various types of cancers including HCC. It plays a role in cell proliferation through activation of cyclin-dependent kinases [15]. It encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein and promotes progression through the G1-S phase of the cell cycle [16]. Caspase-3 is a cysteine protease that plays an important role in the activation of cascade of caspases responsible for apoptosis execution [17]. During programmed cell death, activation of caspase-3 induces proteolysis of DNA repair proteins, cytoskeletal proteins, and the inhibitor of caspase-activated deoxyribonuclease [18]. Our results suggested that these apoptosis-related proteins might be involved in the effects of *NET-1* knockdown on HCC cells.

We checked the PI3K/Akt pathway to further explore the underlying molecular mechanism. The PI3K/Akt pathway is an intracellular signaling pathway that plays a critical role in regulating metabolism, proliferation, growth and apoptosis of a wide range of cancer cell types including HCC [19]. Aberrant PI3K/Akt signaling has been found in HCC by epidemiological and experimental studies [20]. In the present study, we found that siRNA-mediated knockdown of *NET-1* significantly decreased the expression of Akt, suggesting that the PI3K/Akt pathway might contribute to the effect of *NET-1* knockdown on HCC proliferation and apoptosis. In conclusion, our analyses suggest that knockdown of *NET-1* effectively inhibits proliferation and promotes apoptosis of HCC cells by regulating the expression of apoptosis-related proteins including *Bax*, *Bcl-2*, *cyclin D1*, and caspase-3, and the PI3K/Akt signaling pathway.

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