Inhibition of NET-1 suppresses proliferation and promotes apoptosis of hepatocellular carcinoma cells by activating the PI3K/AKT signaling pathway

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Received June 22, 2017; Accepted July 20, 2018

DOI: 10.3892/etm.2019.7211

Abstract. The present study aimed to elucidate the underlying mechanism of neuroepithelial cell transforming 1 (NET-1), a member of the Ras homolog gene family, in hepatocellular carcinoma (HCC). To determine the association between the expression of NET-1 and the proliferation and migration of MHCC97-H cells, the cells were transfected with NET-1 small interfering (si)RNA and si negative control. Following transfection with NET-1 siRNA, the proliferation rate of MHCC97-H cells decreased significantly and the percentage of apoptotic cells increased. The HCC cell line MHCC97-H was used in the present study as it exhibited an increased expression level of NET-1 compared with the MHCC97-L cell line. Expression levels of apoptosis-associated proteins including apoptosis regulator Bax (Bax), cyclinD1, apoptosis regulator Bcl-2 (Bcl-2) and caspase-3 were determined. Expression levels of phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) and their phosphorylated forms were also measured by western blotting. Following NET-1 knockdown, the expression of Bax and cyclinD1 decreased, the expression of Bcl-2 and caspase-3 increased, and the PI3K/AKT signaling pathway was inhibited. The results of the present study suggest that inhibition of NET-1 can suppress the progression of HCC by targeting the PI3K/AKT signaling pathway. NET-1 expression level in HCC cells increased compared with normal liver cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the common malignant tumors worldwide (1). Globally, there are more than 500,000 new cases each year and about 1 million HCC-associated cases of mortality (2-6). Approximately 40-50% of global HCC cases occur in China and HCC is the second most malignant tumor in China (7-10). Although there are a number of methods of treatment for HCC, they are ineffective for achieving sustained remission (11). Invasion, metastasis and postoperative recurrence are the primary causes leading to the mortality of patients with HCC (12). The processes associated with invasion and metastasis of HCC are complex and involve multiple molecular interactions and multiple-level cross regulation of signal transduction pathways (13,14). Therefore, research on the mechanisms of invasion and metastasis of HCC is important to increase the clinical curative effects and improve the survival rate of patients.

Neuroepithelial cell transforming 1 (NET-1), a member of Ras homolog gene family, was identified in 2000 by Serru *et al* (15) and reported to serve a role in signaling pathways, including ERK1/2 and PI3K/Akt1, which may be regulated by NET-1 as well as cell adhesion, proliferation and differentiation (16,17). A study also demonstrated that the inhibition of NET-1 could suppress the activation of ERK1/2 and PI3K/Akt1 signaling (18). Previous studies also indicated that the abnormal expression of NET-1 is associated with numerous types of cancer, including lung, colorectal, gastric and breast cancer (19,20). Shen *et al* (21) reported that NET-1 mRNA is expressed at very low levels in normal liver tissues and highly expressed in HCC tissues, suggesting that this protein may serve as a biomarker in the early diagnosis of liver cancer. Expression of NET-1 is closely associated with the lymphatic and distant metastasis in non-small cell lung cancer (22). One study revealed that inhibition of NET-1 in HCC was associated with the tumor node metastasis stage (23). Therefore, the authors of the present study hypothesized that NET-1 may serve an important role in HCC.

The present study aimed to determine the association between the expression of NET-1 and HCC. The mRNA expression levels of NET-1 in HCC cell lines and a normal liver cell line were compared and the cell line with the highest expression level of NET-1 was selected. The selected cells were transfected with NET-1 small interfering (si)RNA and si negative control (NC), and the proliferation rate and apoptosis of cells were determined. The expression of apoptosis-associated proteins was also determined to elucidate the molecular mechanism of NET-1 in HCC.

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Key words: neuroepithelial cell transforming 1, hepatocellular carcinoma, proliferation, phosphoinositide 3-kinase, protein kinase B

Materials and methods

Cell culture. Human HCC cell lines MHCC97-L and MHCC97-H, and a normal liver cell line L-02 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell lines stored in -80˚C liquid nitrogen was recovered, inoculated, cultured and digested to obtain single cell suspension. Cells were routinely cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37˚C.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, 1 ml of TRIzol was added and each sample was homogenized at 4˚C for 10 min. Subsequently, the lysates were transferred into 1.5 ml Eppendorf (EP) tubes (Eppendorf, Hamburg, Germany). Following shaking for 15 min, the EP tubes were centrifuged at 12,000 x g and 4˚C for 15 min. The supernatant was transferred into new EP tubes and mixed with isopycnic isopropanol for 15 sec. Subsequently, the mixture was centrifuged at 12,000 x g and 4˚C for 10 min, and the supernatant was discarded. The precipitate was washed with 75% ethanol twice and dried. Then, the dried precipitate was dissolved in 30 μl DEPC-treated (0.1%) water (Thermo Fisher Scientific, Inc.) and quantified by a NanoDrop 1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, Delaware, USA) and the RNA solution was stored at -80˚C for further use. Genes were amplified using specific oligonucleotide primers for NET-1 and GAPDH, which was used as the internal control. The forward and reverse primers are listed in Table I. The first strand of cDNA was synthesized by RevertAid First strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) at 42˚C for 10 min. SYBR® Green Real-Time PCR Master mixes (Takara Bio, Inc., Otsu, Japan) and a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland) were utilized to perform a qPCR analysis. The following thermocycling conditions were used for the PCR: 55˚C for 30 min, initial denaturation for 15 min at 95˚C; 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec, 72˚C for 30 sec. The expression level was normalized using GAPDH small nuclear RNA and expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (23).

Western blotting. Cells were seeded into a six-well plate at a density of $5x10^5$ cells/well. A total of 24 h after seeding, the medium was discarded and cells were rinsed 3 times with ice-cold PBS. Subsequently, cells were lysed with radioimmunoprecipitation assay buffer at 4˚C for 15 min and centrifuged at 12,000 x g at 4˚C for 10 min. The precipitation was discarded and the protein extract in the supernatant was quantified by a BCA kit (Thermo Fisher Scientific, Inc.). The supernatants were collected and boiled at 95˚C with an equal volume of loading buffer for 10 min. Subsequently, a total of 12 μg of protein was loaded into 4% spacer and 12% separation gel for SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Hybond, Inc., Escondido, CA, USA). The membranes were blocked with 5% skimmed milk dissolved in Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature. Subsequently, the membranes were rinsed with TBST twice and incubated with primary antibodies, including NET-1 (cat. no. ab5914), Bax (cat. no. ab32503), Cyclin D1 (cat. no. ab134175), Bcl-2 (cat. no. ab32124), Caspase-3 (cat. no. ab13585), PI3K (cat. no. ab86714), p-PI3K (cat. no. ab182651), AKT (cat. no. ab8805), p-AKT (cat. no. ab81283) and GAPDH (cat. no. ab9485; all 1:1,000; Abcam, Cambridge, MA, USA) dissolved in 5% bovine serum albumin (Abcam) at room temperature for 1 h. Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies (cat. no. ab6721; 1:10,000, Abcam) at room temperature for 1 h. Protein bands were visualized using the EZ-ECL Chemiluminescence Detection kit for horseradish peroxidase (Biological Industries, Kibbutz Beit Haemek, Israel).

Cell transfection. A total of $1x10^3$-$1x10^4$ cells/well were seeded in 96-well plates. NET-1 overexpression or control vector plasmids (0.2 μg; both Genentech USA, Inc., South San Francisco, CA, USA) were transfected into cells using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The NET-1 siRNA or scramble control siNC (10 pmol) was synthesized and modified chemically by Invitrogen (Thermo Fisher Scientific, Inc.) using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Inc.). Following 72 h of transfection, cells were harvested for proliferation and apoptosis assays.

Flow cytometry assay. Apoptosis and cell cycle of MHCC97-H cells were detected using flow cytometry kit (cat. no. Apobrdu-1KT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Briefly, MHCC97-H cells at a logarithmic growth phase were seeded in a 96-well plate at a density of $2x10^3$ cells/well and maintained in RPMI 1640 medium (cat. no. SH30809.01; Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (cat. no. AD17321268; Invitrogen; Thermo Fisher Scientific, Inc.) for 16 h at 37˚C. Following cell transfection with control plasmids, control siRNA or NET-1-siRNA for 72 h, the cells were rinsed twice with PBS and counted. A total of 5-$10x10^4$ cells were collected and centrifuged at 2,000 x g for 5 min at 4˚C. Subsequently, cells were resuspended with and incubated for additional 10 min at 37˚C. Centrifugation at 2,000 x g for 5 min at 4˚C was performed and the cells were resuspended in PBS containing 10 μl propidium iodide in the dark for 30 min at room temperature. Finally, apoptosis was measured using a flow cytometer and CellQuest software (version 3.3; BD Biosciences, San Jose, CA, USA).

Cell proliferation assay. Cells were seeded into 96-well plates at a density of $5x10^4$ cells/well the day prior to transfection. Following transfection, cells were seeded in a 96-well plate at a density of $2x10^3$ cells/well. Proliferation of cells was determined using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene name	Primer sequences (5'-3')	
	Forward	Reverse
Neuroepithelial cell transforming 1	GAGCCAAGCAATAAAAGAGTTCG	TGGGACTGTTGACCTGCTAGA
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

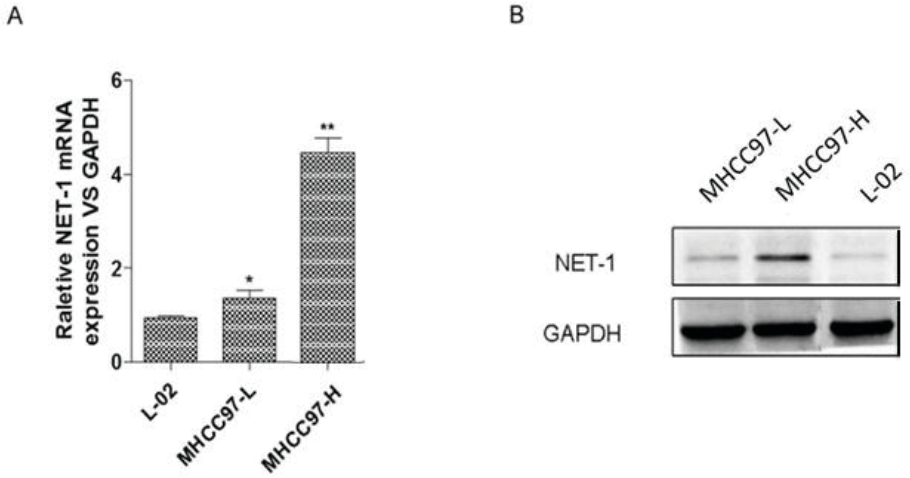

Figure 1. NET-1 expression in HCC cell lines and normal liver cells. (A) Relative mRNA expression levels of NET-1 in HCC cell lines MHCC97-L and MHCC97-H, and normal liver cell line L-02 were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05 and **P<0.01 vs. the L-02 cells. (B) Protein expression levels of NET-1 in HCC cell lines MHCC97-L and MHCC97-H, and normal liver cell line L-02 were determined by western blotting. NET-1, neuroepithelial cell transforming 1; HCC, hepatocellular carcinoma.

the manufacturer's protocol at 12, 24 and 48 h of culture. The optical density (OD) was measured at a wavelength of 450 nm.

Statistical analysis. Data were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard deviation. All experiments were performed in triplicate. Groups were compared using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Screening for HCC cells with high expression of NET-1. To study the effect of NET-1 on HCC, the present study determined the relative mRNA and protein expression levels of NET-1 in HCC cell lines MHCC97-L and MHCC97-H and in a normal liver cell line L-02 using RT-qPCR and western blotting, respectively. The results of the RT-qPCR assay indicated that the expression levels of NET-1 were significantly elevated in MHCC97-L and MHCC97-H cells compared with the L-02 cell line. Specifically, the MHCC97-H cell line exhibited the highest expression of NET-1 among these cell lines (Fig. 1A). Furthermore, western blotting indicated that protein expression of NET-1 increased in MHCC97-L and MHCC97-H cells compared with the L-02 cell line, and MHCC97-H exhibited the highest expression level among these cell lines (Fig. 1B). Therefore, MHCC97-H cells were selected for further analysis.

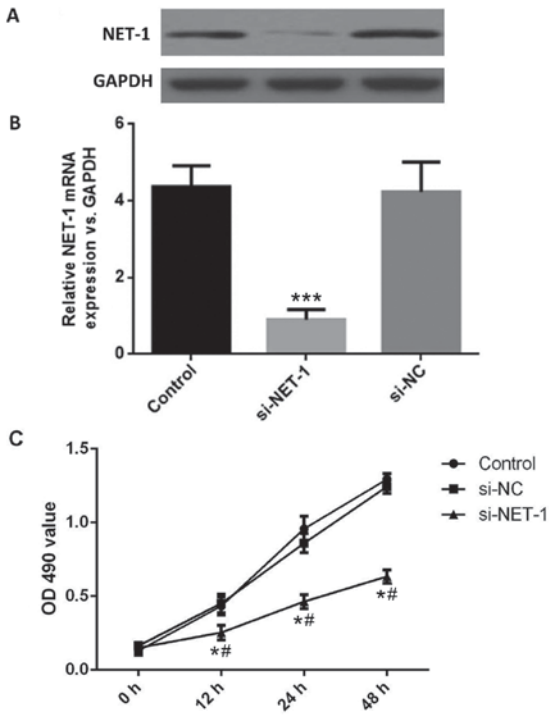

Figure 2. Verification of transfection efficiency and cell proliferation assays. (A) Protein and (B) mRNA expression of NET-1 in differentially treated cells. (C) The OD 490 value of MHCC97-H cells following transfection with NET-1 siRNA or siNC. *P<0.05, ***P<0.001 vs. control; $^{\#}$P<0.05 vs. si-NC. NET-1, neuroepithelial cell transforming 1; si, small interfering RNA; si-NC, small interfering RNA negative control.

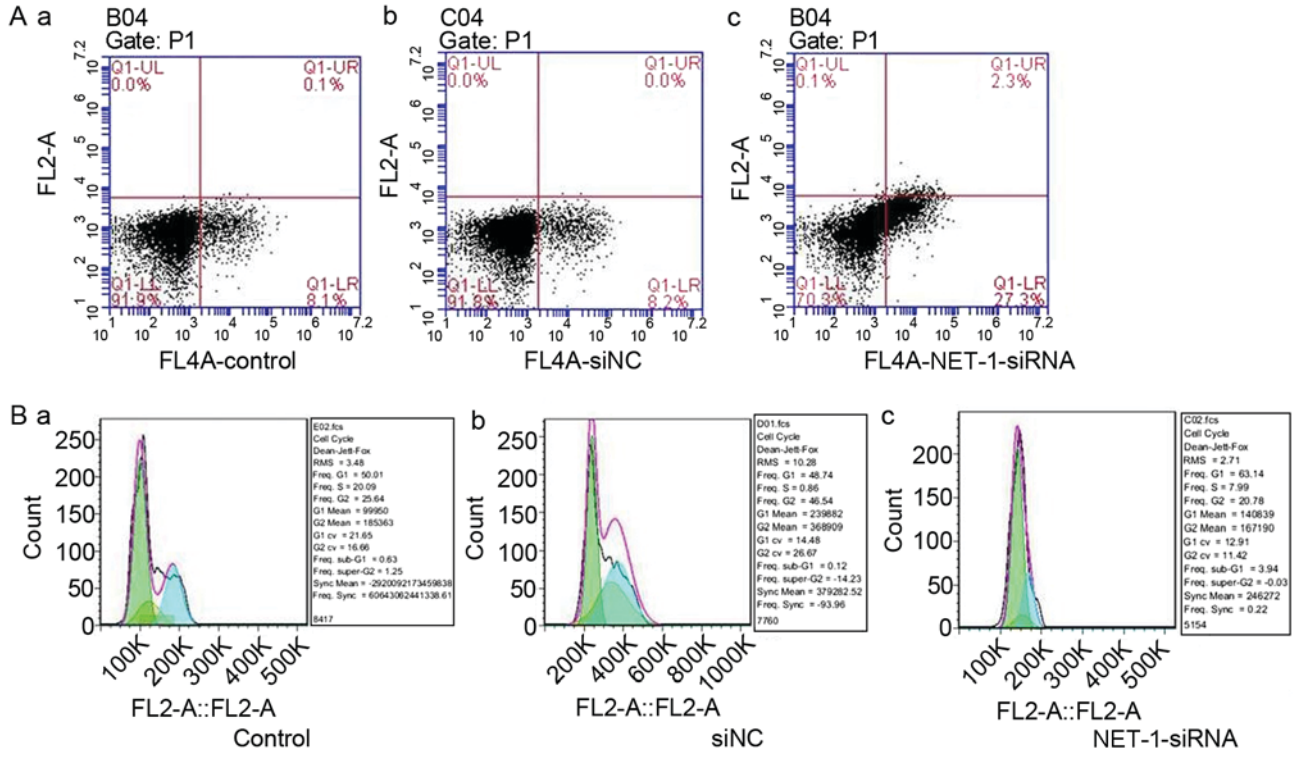

Figure 3. Knockdown of NET-1 promotes hepatocellular carcinoma cell apoptosis. (A) Apoptotic percent of MHCC97-H cells in the (a) control, (b) siNC and (c) NET-1 siRNA groups. (B) Cell cycle distribution of MHCC97-H cells in the (a) control, (b) siNC and (c) NET-1 siRNA. siRNA, small interfering RNA; siNC, small interfering RNA negative control; NET-1, neuroepithelial cell transforming 1.

Knockdown of NET-1 inhibited the proliferation of HCC cells. Following transfection with si-NET-1, the mRNA and protein expression of NET-1 was successfully downregulated in cells compared with the control (Fig. 2A and B). The OD value of MHCC97-H cells was determined by CCK-8. The viability of MHCC97-H cells in the si-NET-1 group was significantly decreased after 12, 24 and 48 h compared with the control groups (Fig. 2C), suggesting the inhibition of NET-1 could inhibit the proliferation of HCC.

Knockdown of NET-1 promotes HCC cell apoptosis. To study the effect of NET-1 on HCC, the apoptotic rate and cell cycle of MHCC97-H cells were determined using flow cytometry. The apoptotic percent of MHCC97-H cells increased following the knockdown of NET-1 compared with the control and si-NC groups (Fig. 3A). Furthermore, cell cycle of MHCC97-H cells was arrested at the G1/S phase following transfection with NET-1 siRNA (Fig. 3B).

Knockdown of NET-1 influences the expression of apoptosis-associated proteins and the activity of the PI3K/AKT signaling pathway. To further reveal the underlying mechanism of NET-1 in HCC, expression levels apoptosis-associated proteins were determined by western blotting. The expression levels of Bax and cyclinD1 in MHCC97-H cells decreased following the knockdown of NET-1, while the expression of Bcl-2 and caspase-3 increased (Fig. 4A). The activity of the PI3K/AKT signaling pathway was also determined when PI3K expression was reduced by the NET-1 siRNA. There was no apparent difference identified in the activity of PI3K, however, the expression of p-AKT decreased following transfection with si-NET-1 (Fig. 4B).

Discussion

HCC is the most common type of primary liver cancer and has been reported to be the fifth most common cancer worldwide (10). The incidence of HCC has increased worldwide and this disease is characterized by geographic risk factor and diagnosis differences (24). There remains no standard effective therapy for patients with HCC. This type of carcinoma is associated with a high degree of vascular invasion and metastasis, and poor prognosis (25). Numerous factors contribute to the invasion and metastasis of HCC. Twist-related protein 1 is a regulator of EMT-mediated invasion and metastasis, which affects the expression of E-cadherin (26). As a pro-inflammatory cytokine, interleukin (IL)-17A is frequently involved in the pathology of inflammatory diseases and regulation of tumor microenvironment (27-29). A previous study reported that IL-17A promoted the metastasis of HCC (30). As a tumor suppressor, microRNA-122 was reported to regulate the intrahepatic metastasis of HCC (31). It has also been demonstrated that NET-1 exhibits higher expression levels in HCC cells compared with normal liver cells, suggesting that NET-1 may serve a role in HCC (21).

In the present study, the mechanism of NET-1 in the invasion and metastasis of HCC was investigated *in vitro*. Relative mRNA expression of NET-1 was determined using RT-qPCR in MHCC97-H and MHCC97-L cells with different metastasis potentials (32,33) and normal liver cell line L-02. The results indicated that the expression of NET-1 was upregulated in HCC cell lines compared with the normal liver cell line, which may contribute to the metastasis and invasion of HCC. The MHCC97-H cell line exhibited the highest expression level

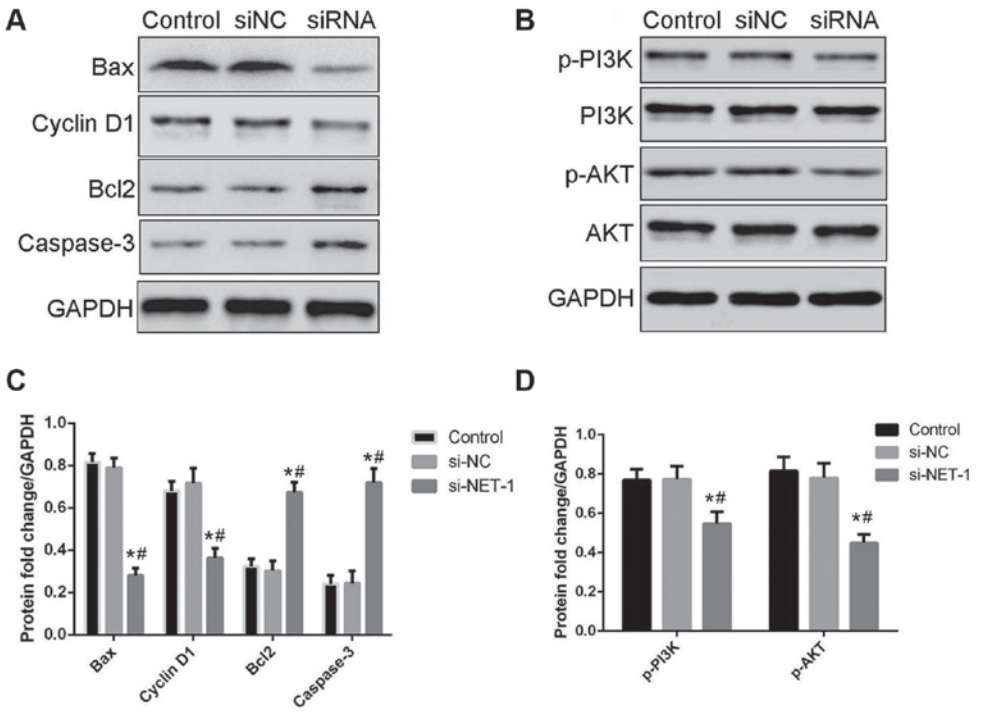

Figure 4. Knockdown of NET-1 influences the expression of apoptosis-associated proteins and the activity of the PI3K/AKT signaling pathway. (A) Protein expression of Bax, cyclinD1, Bcl-2 and caspase-3. (B) Protein expression of p-PI3K, PI3K, p-AKT and AKT. (C) Quantification of the protein levels of Bax, cyclinD1, Bcl-2 and caspase-3. (D) Quantification of the protein levels of p-PI3K and p-AKT. *P<0.05 vs. control; #P<0.05 vs. siNC. Bax, apoptosis regulator Bax; Bcl-2, apoptosis regulator Bcl-2; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; siNC, small interfering RNA negative control; siRNA, small interfering RNA targeting neuroepithelial cell transforming 1.

of NET-1 and was therefore selected for subsequent experiments. NET-1 was knocked down in MHCC97-H cells and proliferation, cell cycle progression and apoptosis were determined. The results indicated that si-NET-1 could decrease the proliferation of MHCC97-H cells. Furthermore, the apoptotic percent of MHCC97-H cells was elevated following the knockdown of NET-1. In addition, cell cycle was arrested at the G1/S phase in the si-NET-1 group of MHCC97-H cells. Shen *et al* (21) demonstrated that the expression of NET-1 was associated with the proliferation, metastasis and clinical stages of HCC. Chen *et al* (34) reported a strong correlation between the expression level of NET-1 and HCC pathological grading. Therefore, in the present study it was hypothesized that NET-1 may serve a role in promoting proliferation and suppressing apoptosis of HCC.

To further elucidate the molecular mechanisms of NET-1, the expressions levels of Bax, cyclinD1, Bcl-2 and caspase-3 were determined. The expression levels of Bax and cyclinD1 decreased in the si-NET-1 MHCC97-H cells, while the expression levels of Bcl-2 and caspase-3 increased compared with the controls. As a pro-apoptotic member of the Bcl-2 family, Bax shares highly conserved domains with Bcl-2 and serves a role in regulating programmed cell death (35). Dysfunction of the p53/Bax/caspase-3 apoptosis signaling pathway promotes carcinogenesis (36). Furthermore, a balance between Bax and Bcl-2 is also involved in cancer therapeutic resistance (37), as well as proliferation, invasion, adhesion and metastasis of cancer cells (38). In a human breast cancer line, overexpression of Bcl-2 enhanced the metastatic ability (39). Cyclin D1 is a proto-oncogene abnormally overexpressed in several cancers, including breast and prostate cancers, which promotes cell proliferation via activation of cyclin-dependent kinases (40). Cyclin D1 may act as a subunit of a holoenzyme to phosphorylate and inactivate the retinoblastoma protein, and promote cell cycle progression to the G_2 phase of the cell cycle (41). Apoptosis is an important mechanism of cell death regulation which serves a role in eliminating infected, damaged and other undesirable cells from tissues (42,43). Caspase-3 is the main executor of apoptosis in cells (44). During programmed cell death, activation of caspase-3 leads to proteolysis of DNA repair proteins and cytoskeletal proteins to alter the morphology and DNA of cells (45). Dysregulation of caspase-3 was reported in several malignancies (46-48) and overexpression of this protein was reported in HCC (49).

To further explore the molecular mechanism of NET-1 in HCC, the activity of the PI3K/AKT signaling pathway was determined. The results indicated that there was no apparent difference identified in the expression of PI3K, however, the expression of AKT was downregulated following knockdown of NET-1. The PI3K/AKT signaling pathway serves an important role in mediating survival signals in a number of neuronal cell types (50). AKT and AKT-dependent signaling pathways, including glycogen synthase kinase-3β (51), PI3K (52) and mitogen-activated protein kinase (53) signaling pathways serve critical roles in the pathogenesis of degenerative diseases and cancers (51), including apoptosis, metabolism, cell proliferation and cell growth (50). Epidemiological and experimental studies reported that abnormally activated PI3K/AKT pathway is involved in the initiation and maintenance of cancer (52-55). In addition, the PI3K/AKT signaling pathway has also been

confirmed to participate in leptin-mediated promotion of invasion and migration of HCC (56). Therefore, these studies verified the reasons why NET-1 promotes proliferation and inhibits apoptosis of HCC cells.

In conclusion, inhibition of NET-1 can suppress proliferation and promote apoptosis of HCC cells by activating the PI3K/AKT signaling pathway and increasing the expression levels of apoptosis-associated proteins.

Acknowledgements

Not applicable.

Funding

The current study was supported by the Key Development Projects in Shandong (grant no. 2018GSF118191) and Shandong Medical Science and Technology Development Program (grant no. 2017WS321).

Availability of data and materials

All data and materials in the present study were available when proper request to the authors.

Authors' contributions

XS conceived of and designed the present study, collected and consolidated the data, analyzed and interpreted the data, and wrote the manuscript. MW conceived of and designed the current study, and collected and consolidated the data. FZ conceived of and designed the current study, analyzed and interpreted the data, and wrote the manuscript. XK conceived of and designed the current study, analyzed and interpreted the data, and wrote the manuscript.

Ethics approval and consent to participate

Ethical approval for cell culturing was given by the Medical Ethics Committee of Linyi People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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