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Original Research

NET-1 promotes invasion, adhesion and growth of hepatocellular carcinoma by regulating the expression of BAX, caspase 3, caspase 8 and BCL2

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Abstract: Dysregulation of neuroepithelial transforming gene-1 (NET-1) has been shown in hepatocellular carcinoma (HCC) patients. We aimed to evaluate the influence of NET-1 on HCC invasion, adhesion and growth. *In vitro* cellular functional assays including invasion and adhesion were performed to evaluate the effects of knockdown and overexpression of NET-1. HCC cells were transplanted into nude mice, and tumor growth was assessed. BAX, caspase 3, caspase 8 and BCL2 protein levels were detected by western blot. After transfection with NET-1 siRNA, NET-1 positive ratio in HCC cells significantly decreased. Cell invasion and adhesion assay showed that knockdown of NET-1 reduced the invasion and adhesion ability of HCC cells, whereas overexpression of NET-1 increased the ability. The evaluation of tumor growth revealed that NET-1 knockdown significantly decreased tumor volume and weight, while NET-1 overexpression promoted tumor growth in nude mice. Western blot showed that NET-1 knockdown increased BAX, caspase 3 and caspase 8 expression but decreased BCL2 expression, whereas NET-1 overexpression significantly down-regulated BAX, caspase 3 and caspase 8 expression but increased BCL2 expression. Our data suggest that NET-1 promotes HCC invasion, adhesion and growth by regulating BAX, caspase 3, caspase 8 and BCL2 expression.

Key words: Growth; Hepatocellular carcinoma; Invasion; NET-1.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer mortality, killing about 700,000 individuals each year worldwide (1). It is a major global health problem and causes a huge economic burden in both developed and developing countries. Surgical resection and liver transplantation are considered the only cures for HCC, but benefit aproximately 10-15% of patients (2).Hence, there is an urgent need to develop novel pharmacological treatment strategies for this devastating disease (3). Invasion and adhesion are essential steps for HCC progression. The process of invasion and adhesion is complex, involving interaction of multiple molecules (4). Elucidating the molecules and underlying mechanisms for invasion and adhesion is crucial for improving our understanding of HCC pathophysiology.

Neuroepithelial transforming gene 1 (NET-1) is a RhoGEF specific for the RhoA subfamily of small G proteins. Rho proteins play an important role in regulating angiogenesis, invasion, and metastasis of human cancers (5). Overexpression of NET-1 has been reported in a variety of human cancers, including HCC, gastric cancer, and lung cancer (6-8). The study by Shen et al found that NET-1 mRNA expression was significantly up-regulated in HCC tissues, and it was positively correlated with proliferating cell nuclear antigen (PCNA) expression and TNM stages (9). In addition, compared with HCC patients with negative-low NET-1 expression, patients with the moderate-strong NET-1 positive expression had worse disease-free survival and overall survival (7). Short interfering RNA (siRNA)-mediated knockdown of NET-1 in HCC cells effectively suppressed cell proliferation and migration (10,11). These lines of evidence suggest that NET-1 may be an important regulator in the development and progression of HCC. In the present study, we aimed to evaluate the influence of NET-1 on HCC invasion, adhesion and growth using *in vitro* and *in vivo* studies.

Materials and Methods

Cell culture

We obtained human HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, and HepG2, and the normal liver cell line L-02 from the Liver Cancer Institute of Fudan University, China. The cells were cultivated in RPMI-1640 medium containing 10% inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO2.

Cell Transfection

MHCC97-L and MHCC97-H cells (1.5×10^5 cells per well) were plated in 6-well plates (Nunc, Rochester, NY, USA). When the cells reached 70% confluence, they were transfected with the plasmids using Lipofectamine 2000™ (Invitrogen) in accordance with the manufac-

turer's protocol. At 48 h posttransfection, images were analyzed by direct fluorescence microscopy (Olympus, Beijing, China). The cells were divided into 4 groups: MHCC97-H, MHCC97-H-inhibitor, MHCC97-L and MHCC97-L-mimic.

Western blotting

Cells were cultivated in 6-well plates (5×10^5 cells each plate) for 24 h. Protein was extracted from cells using radioimmunoprecipitation assay (RIPA) buffer. After determing protein concentrations, samples were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membrane (Hybond, Escondido, CA, USA). The membranes were placed in Tris-buffered saline Tween-20 (TBST) containing 5% skimmed milk powder and blocked for 1h at room temperature. Following washing, the membranes were incubated with specific primary antibodies diluted with 5% bovine serum albumin (BSA). The protein bands were visualized by EZ-ECL chemiluminescence detection kit for horseradish peroxidase (HRP) (Biological Industries, Beit-Haemek, Israel).

Immunohistochemistry (IHC)

IHC was used to detect the level of NET-1 in HCC cells transfected with NET-1-inhibitor and NET-1-mimic. Sections were deparaffinized, rehydrated, and blocked with methanolic 3% hydrogen peroxide. The slides were immersed in 10 mm citrate buffer, pH 6.0 and heated in a 1200W microwave oven at the highest power setting. Evaporated liquid was replenished at 5 minutes, then the slides were heated at high power for an additional 5 minutes. The slides were left in the buffer for an additional 10 minutes before being removed. Immunostaining was performed by hand using the prediluted antibody solution and a 30 minute incubation. After washing, antibody binding was visualized using the avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlington, VT), followed by incubation with 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin.

Invasion assay

The invasive potential of MHCC97-L and MHCC97-H cells was determined by an invasion assay using polycarbonate membranes (8.0-μmpore size) in the upper half of 24-well Transwell culture chambers coated with Matrigel (Costar). At 24 h after transfection, HCC cells (1×10^5) were suspended with 100 μL serum-free RPMI-1640 medium and placed in the upper chamber. The lower compartment of the chamber was filled with 500 μL RPMI-1640 medium containing serum. After 24 h incubation in 5% CO2 at 37°C, nonmigratory cells on top of the filters were gently removed with cotton swabs. The invading cells on the underside of the filter were fixed for 10 min in 10% formaldehyde, stained with 1% crystal violet for 5 min, and washed with PBS. The number of cells was quantified by counting the cells in at least 5 random fields per filter (magnification, ×200).

Cell adhesion experiment

Fn (10 μg/ml) was added into 96-well plates, put aside overnight and washed with PBS for three times. Cells at logarithm phase were collected and cultured in serum-free medium at a concentration of 5×10^5/mL. Cells were inoculated into a 96-well plate with 10000 cells/well. After 24 h of cultivation at 37°C, the adherent cells were washed by PBS. Then, 20 uL of MTT solution (0.5% MTT) was added to each well and cultivated at 37°C with 5% CO_2 for 4 h. When culture medium was drawn away, 150 uL of DMS was added into each well and the plate was shaked for 10 min to make crystal dissolve well. The optical density (OD) at 490 nm was read using a microplate reader.

Evaluation of tumor growth

Female nude mice (SPF grade) weighing 20 g were purchased from Shanghai Slack Laboratory Animal Co., LTD (Shanghai, China). MHCC97-H cells, MHCC97-H cells transfected with NET-1 siRNA, MHCC97-L cells and MHCC97-L cells transfected with myc-NET-1 were transplanted into nude mice. After subcutaneous transplantation, tumor bodies were taken out and immersed into 4°C sterile saline solution for use. Tumor growth in nude mice was evaluated every 3 weeks.

Statistical analysis

Categorical data were expressed in proportion, while continous data were presented as means±SD. A *P* value of < 0.05 was considered statistically significant.

Results

Knockdown and overexpression of NET-1 in HCC cells

RT-PCR and western blot analysis confirmed that the level of NET-1 in HCC cell lines was much higher than that in the normal liver cell line L-02 (not shown). The highest level of NET-1 was observed in MHCC97-H cells, whereas the lowest level was found in MHCC97-L cells among the HCC cell lines. Hence, MHCC97-H cells were selected for further study. IHC was used to detect the level of NET-1 in HCC cells transfected with NET-1-inhibitor and NET-1-mimic. The results demonstrated that the positive ratio of NET-1 in the inhibitor-transfected MHCC97-H cells decreased si-

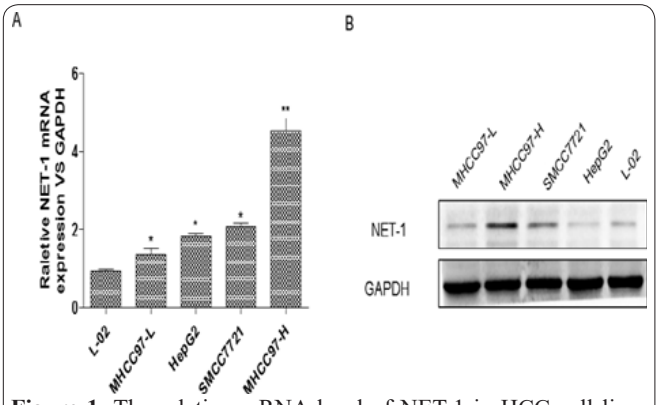

Figure 1. The relative mRNA level of NET-1 in HCC cell lines MHCC97-L, MHCC97-H, SMCC7721, HepG2 and normal liver cell lines L-02 by RT-PCR. **P<0.05* and ***P<0.01*, compared with L-02 cells, mRNA level of NET-1 in HCC cell lines had statistical difference. B. The protein expression of NET-1 in HCC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

gnificantly compared with non-transfected MHCC97-H cells (Figure 1A). In addition, the positive ratio of NET-1 in MHCC97-L cells transfected with NET-1 mimic increased significantly compared with non-transfected MHCC97-L cells (Figure 1A).

Knockdown of NET-1 inhibited the invasion of HCC cells

Invasion assay showed that the number of invasive cells in the MHCC97-H-inhibitor group was much smaller than that in the MHCC97-H group (Figure 1B). In contrast, the number of invasive cells in the MHCC97-L-mimic group was larger than that in the MHCC97-L group (Figure 1B). The results of cell adhesion assay revealed that the adherence rate of HCC cells in the MHCC97-H-inhibitor group was significantly lower than that in the MHCC97-H group, whereas the value in the MHCC97-L-mimic group was markedly higher than that in the MHCC97-L group (Figure 1C).

Knockdown and overexpression of NET-1 affected HCC tumor volume and weight

The tumor volume in the MHCC97-H-inhibitor group was markedly smaller than that in the MHCC97-H group, whereas the volume in the MHCC97-L-mimic group was significantly larger than that in the MHCC97-L group (Figure 2A). Tumor weight was also evaluated. The evaluation showed that tumor weight in the

MHCC97-H-inhibitor group was markedly lower than that in the MHCC97-H group, whereas the weight in the MHCC97-L-mimic group was significantly higher than that in the MHCC97-L group (Figure 2B).

Knockdown and overexpression of NET-1 influenced the expression of apoptosis-related proteins

We assessed the protein levels of BAX, caspase 3, caspase 8 and BCL2 to investigate the underlying mechanisms by which NET-1 affected HCC cells. Western blot analysis revealed that the protein expression of BAX, caspase 3 and caspase 8 in the MHCC97-H-inhibitor group was markedly up-regulated in comparison to the MHCC97-H group, whereas the expression of these proteins was reduced in the MHCC97-L-mimic group compared with the MHCC97-L group. BCL2 protein expression in the MHCC-H-inhibitor group was markedly lower than that in the MHCC97-H group, whereas its expression was dramatically increased in the MHCC97-L-mimic group compared with the MHCC97-L group (Figure 3).

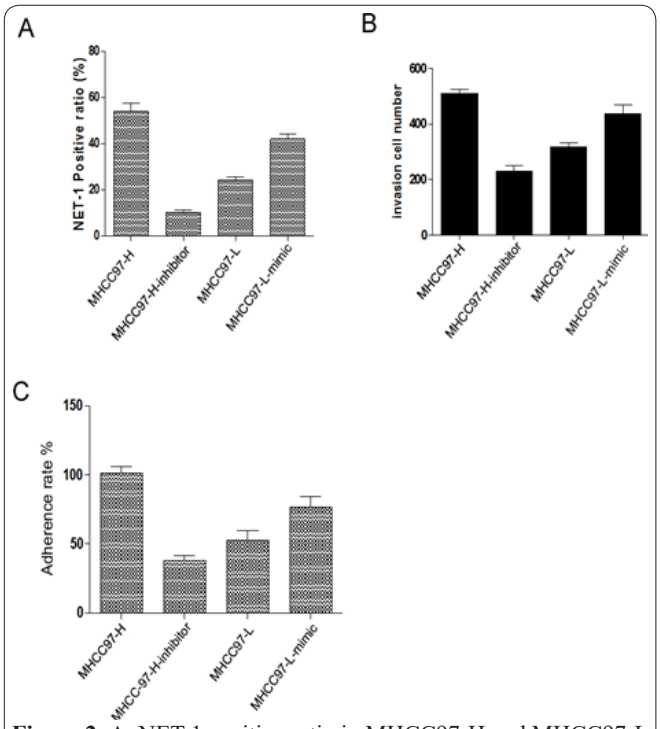

Figure 2. A. NET-1 positive ratio in MHCC97-H and MHCC97-L cells after knockdown or overexpression of NET-1. B. The number of invasive cells in MHCC97-H, MHCC97-H-inhibitor, MHCC97-L and MHCC97-L-mimic groups. C. Evaluation of the effects of knockdown and overexpression of NET-1 on adherence rate of HCC cells.

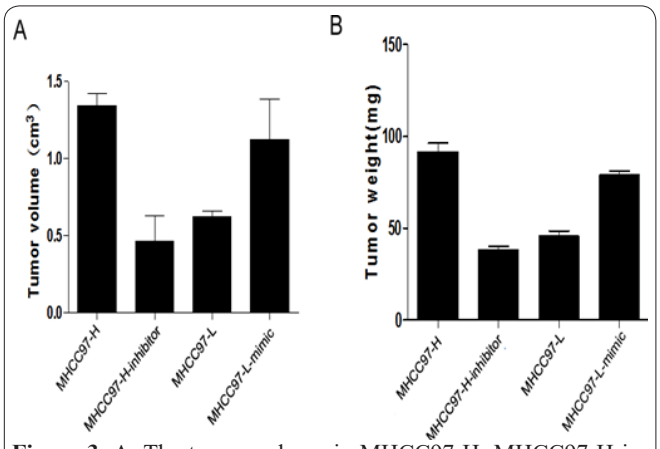

Figure 3. A. The tumor volume in MHCC97-H, MHCC97-H-inhibitor, MHCC97-L and MHCC97-L-mimic groups. B. The tumor weight in MHCC97-H, MHCC97-H-inhibitor, MHCC97-L and MHCC97-L-mimic groups.

Discussion

HCC accounts for the vast majority of primary liver cancers (85-90%) (12). It is among the top three causes of cancer death in East Asia due to the high prevalence of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. The prognosis of HCC is dismal with a 3-year survival rate of 12.7% and a median survival of 9 months (13). Despite advances in our understanding of the molecular pathways involved in HCC, our therapeutic armamentarium remains limited Therefore, it is urgent to develop more effective pharmacological treatments for HCC.

NET-1 is a well characterised oncoprotein that is involved in cytoskeletal organisation and cancer cell proliferation. Previous studies demonstrated that NET-1 was highly expressed in some human tumor cell lines and tissues. The study by Xu et al showed that NET-1 expression in prostate tumors was much higher than that in normal prostate tissue (14). In addition, NET-1 was overexpressed in HCC, lung cancer and gastric cancer (6-8). In this study, we aimed to explore the influence of NET-1 on HCC cell invasion, adhesion, and tumor growth.

Cell invasion assay showed that knockdown of NET-1 significantly decreased the number of invasive HCC cells, while overexpression of NET-1 markedly increased the number. The adhesion rate of HCC cells decreased after transfection with NET-1 siRNA in the MHCC97-H-inhibitor group, while the adhesion rate

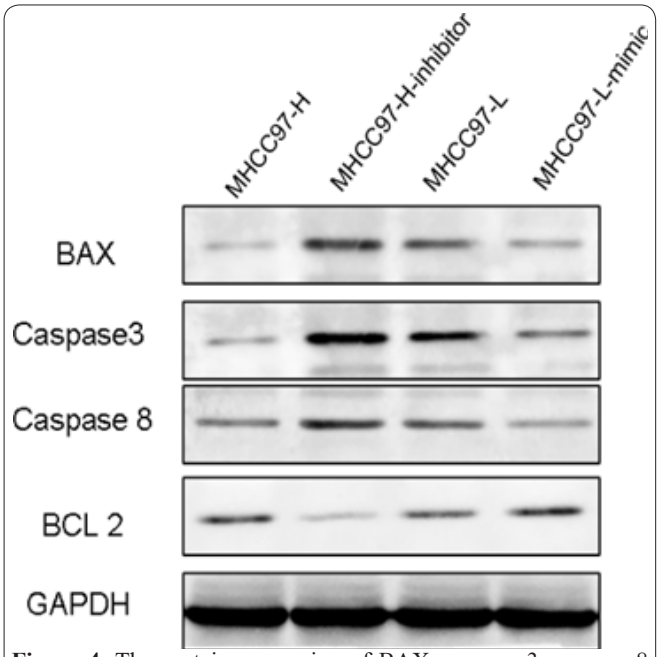

Figure 4. The protein expression of BAX, caspase 3, caspase 8 and BCL2 in MHCC97-H, MHCC97-H-inhibitor, MHCC97-L and MHCC97-L-mimic groups.

increased markedly after transfection with myc-NET-1 in the MHCC97-L-mimic group.

MHCC97-H cells, MHCC97-H cells transfected with inhibitors, MHCC97-L cells and MHCC97-H cells transfected with myc-NET-1 were transplanted to nude mice. We noticed that tumor volume and weight were significantly increased in the

MHCC97-L-mimic group, while they were markedly decreased in the MHCC97-H-inhibitor group. Thus, the results suggested that NET-1 promoted

invasion and adhesion of HCC cells *in vitro*, and enhanced HCC growth *in vivo*. Our results were consistent with previous findings suggesting a role of NET-1 in HCC development (10, 15). In addition, using *in vivo* studies, we provided more important evidence for the promoting effect of NET-1 on HCC growth compared with previous studies, because most of them only performed *in vitro* assays.

BAX is an essential proapoptotic protein whose apoptotic function is antagonized by BCL2 expression (16). The study of Guo et al reported that BAX and BCL2 played an important role in regulating the apoptosis of normal liver and HCC (17). Caspase-3 is a caspase protein that interacts with caspase 8 and caspase 9 and involved in the execution phase of apoptosis (18). It is a crucial component of the apoptotic machinery in many cell types. The expression of caspase 3 was increased in HCC and was related with HCC apoptosis (19). Caspase-8 is a member of the cysteine proteases, which plays a pivotal role in the extrinsic apoptotic signaling pathway via death receptors (20). It is frequently inactivated by the frameshift somatic mutation 1225_1226delTG in HCC (21). BCL2 has been studied intensively owing to its importance in the regulation of apoptosis, tumorigenesis and cellular responses to anti-cancer therapy (22). It is strongly associated with apoptosis of HCC cells (23). In the present study, the protein levels of BAX, caspase 3 and caspase 8 were increased after knockdown of NET-1, whereas they decreased after overexpression of NET-1. In addition, NET-1 knockdown markedly decreased BCL2 protein expression, whereas NET-1 overexpression dramatically up-regulated BCL2 expression. Our analysis suggested that NET-1's promoting effects on HCC invasion, adhesion and growth may in part be attributable to the expression change of these proteins.

In summary, our results suggested that NET-1 promoted HCC invasion, adhesion and growth by influencing the expression of BAX, caspase 3, caspase 8 and BCL2.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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