BNIPL-2 promotes the invasion and metastasis of human hepatocellular carcinoma cells

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Abstract. Enhanced cell migration and invasion play key roles in cancer metastasis. However, the molecules involved in this process are not fully understood. In this study, a full-length human BNIPL-2 (Bcl-2/adenovirus E1B 19 kDa interacting protein 2 like-2) cDNA was transfected into human hepatocellular carcinoma cells with low metastatic potential (MHCC97-L). The *in vitro* and *in vivo* effects of BNIPL-2 on cell invasion and metastasis were examined. *In vitro* analysis showed that the overexpression of BNIPL-2 increases cell invasion and promotes cell migration. The rates of intra-hepatic and pulmonary metastasis in nude mice were also increased. Cdc42 activation assays and immunoblot analysis indicated that the activation of Cdc42 and the upregulation of CD44 were involved in the metastasis of cancer cells. The overexpression of BNIPL-2 promotes the invasion and metastasis of MHCC97-L cells. Thus, BNIPL-2 is a gene related with cancer metastasis.

Introduction

Tumor invasion and metastasis are major obstacles for successful cancer therapy (1). Cancer metastasis is a multi-step process that involves the dissociation of tumor cells from the primary tumor followed by their entry into the circulation, extravasation from the vascular system and proliferation into tumor masses at secondary tissue sites. Failure at any of these steps can block the entire metastatic process (2,3). Determining which proteins are required for cancer metastasis is an important first step in developing therapeutics aimed at limiting the metastasis or dispersal of tumor cells.

Important biological processes in the development of metastasis include the increased migration and invasion of tumor cells. Cell migration is associated with the reorganization of the actin cytoskeleton that triggers changes in cell morphology. The activation of members of the Rho family of GTPases, such as RhoA, Rac1, and Cdc42, controls the dynamics of the actin cytoskeleton. Thus, they represent key regulatory molecules during cell migration (4). BNIPL-2 was identified as a novel member of the BNIPL family by our laboratory and we found that it interacted with Cdc42GAP and suppressed the GAP activity of Cdc42GAP towards Cdc42 (5). According to previous results, the BCH domain (BNIP-2 and Cdc42GAP homology) induces short and long pseudopodia, and is subsequently needed to trigger cell migration (6). As BNIPL-2 has a BCH domain, it could be involved in cell migration and cancer metastasis.

We report here that the overexpression of BNIPL-2 increased cell migration and invasion *in vitro* and promoted the metastasis of hepatocellular carcinoma (HCC) cells *in vivo*. Furthermore, BNIPL-2 promotes the activition of small GTPases such as Cdc42, resulting in the formation of filopodia. Our data show the molecular mechanisms mediating the BNIPL-2-induced migration and metastasis of cancer cells.

Materials and methods

Cells and cell culture. Human hepatocellular carcinoma cells with low metastatic potential (MHCC97-L) were obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (7). The cells were cultured in DMEM medium (Gibco BRL-Life Technologies, Grand Island, NY, USA), containing 10% quality fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 μg/ml strepto-mycin at 37˚C in 5% CO_2.

Transfection. A full-length human BNIPL-2 cDNA was cloned into the pEGFP-N1 plasmid (Clontech Laboratories,

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Inc., Palo Alto, CA, USA). The recombinant was named pEGFP-BNIPL-2. The transfection of plasmid DNAs into MHCC97-L cells was performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Briefly, $1x10^5$ cells were seeded per well into 6-well plates (Corning Inc., Corning, NY, USA). The cells were transfected using 1 μg DNA mixed with 7 μl Lipofectamine in 1 ml DMEM without serum and antibiotics. The cells were incubated for 6 h, and then 1 ml DMEM containing 20% serum was added into each well. The cells were cultured and screened in the medium containing 10% serum and 2 mg/ml G418 (Invitrogen) for at least two weeks till the non-transfected MHCC97-L cells cultured in the controlled wells were all killed. A colony transfected stably with pEGFP-BNIPL-2 was selected, and this was designated as the MHCC97-L BNIPL-2 cells. The stable transfectants of MHCC97-L transfected with the pEGFP-N1 vector control were called MHCC97-L VC.

Immunoblotting. Immunoblot experiments were performed according to standard procedures. Briefly, the cells (MHCC97-L BNIPL-2 or MHCC97-L VC cells) were harvested in lysis buffer [T-PER™ tissue protein extraction reagent (Pierce Chemical Company, Rockford, IL, USA) containing 1% proteinase inhibitor cocktail] and clarified by centrifugation at 10,000 x g. For protein extraction from the tissues, the samples were snap-frozen and immediately homogenized in lysis buffer. Equal amounts of protein (20 μg/lane) were electrophoresed on 10% SDS-PAGE and blotted to the PROTRAN nitrocellulose transfer membrane. The rabbit polyclonal BNIPL-2 antibody was made by our laboratory (5). Monoclonal anti-ß-actin and the anti-CD44 antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Secondary anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunocomplexes were visualized using the SuperSignal west femto maximum sensitivity substrate (Pierce Chemical Company).

Cell invasion assays. The invasive potential was measured by an *in vitro* Boyden chamber assay (8) with some modifications. Briefly, the cells were treated with trypsin-EDTA and counted. MHCC97-L BNIPL-2 or MHCC97-L VC cells ($4x10^4$) in 0.1 ml serum-free DMEM were added to the well of the 8-μm pore membrane Boyden chamber (Costar, Cambridge, MA, USA) coated with Matrigel. The lower chambers contained 10% FBS in DMEM, serving as a chemoattractant. The cells were allowed to invade for 24 h. The cells that had not penetrated the filters were removed by scrubbing with cotton swabs. The chambers were fixed for 20 min at room temperature with 4% formaldehyde in PBS, stained in 0.5% crystal violet for 20 min, and rinsed in water. The cells that migrated to the lower surface of the filter were considered to have invaded the matrix and were counted with a light microscopy.

Wound healing assays. Wound healing assay was performed to study the effect of BNIPL-2 on cell motility, following the previous report with slight modifications (9). In this assay, MHCC97-L BNIPL-2 or MHCC97-L VC cells were grown to confluence in 60-mm dishes, and then the 'wound' was

created by stripping a sharply defined channel using a razor-blade. To prevent growth during migration, the cells were pretreated with 2 mM thymine (Sigma-Aldrich) for 24 h. After band-stripping, the cells were allowed to migrate into the wound for 24 h, fixed with 4% formaldehyde in PBS for 20 min, and then stained with crystal violet. The number of individual cells in the wound was quantitated as the average from six random fields under the microscope.

Xenograft model. A metastasis model was made on mice as described previously (10). Cells ($5x10^6$) (MHCC97-L BNIPL-2 or MHCC97-L VC cells) were subcutaneously injected into the right upper flank regions of experimental mice (4-6 weeks of age, male, BalB/c nu$^+$/nu$^+$, from the Shanghai Cancer Institute). The mice were housed in laminar flow cabinets under specific pathogen-free conditions. Once the subcutaneous tumor reached 1-1.5 cm in diameter, it was removed and cut into pieces of about 1x1x1 mm which were implanted into the liver of each of the 8 nude mice. After 35 days, the animals were sacrificed and autopsied. The mouse whose small tumor colonies around the tumor nodule derived from the implanted tumor tissue at the implantation site were visible to the naked eye, was considered as intrahepatic metastasis. The lungs were sampled for histopathological studies. The mouse whose metastatic HCC cells were found on any slide of the lung sections, was considered as lung metastasis. The animals were treated according to the guidelines established by the American Association for Laboratory Animal Care (AALAC).

Production of GST-PAK-CRIB domain (PAK-CD) fusion proteins. The cloning, expression and purification of the glutathione-S-transferase (GST)-PAK-CD fusion protein (encompassing amino acids 56-272 from PAK1B), containing the Cdc42 binding region from human PAK1B, have been described previously (11). In brief, *Escherichia coli* Top10F' cells were transformed with the GST-PAK-CD construct. The expression of the recombinant protein was induced by the addition of 0.1 mM isopropylthiogalactoside. The cells were harvested, resuspended in PBS containing 1% proteinase inhibitor cocktail, and then sonicated. The cell lysates were centrifuged and the supernatant was incubated with glutathione-coupled Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 30 min at 4˚C. The protein bound to the beads was washed three times in PBS and the amount of fusion protein was estimated using Coomassie-stained SDS gel.

Cdc42 activation assays. Cells ($5x10^6$) were lysed in lysis buffer (T-PER™ tissue protein extraction reagent containing 1% proteinase inhibitor cocktail), clarified by centrifugation, and incubated with 200 μg bacterially produced GST-PAK-CD fusion protein for 60 min. The proteins bound to the fusion protein were precipitated by the use of glutathione-Sepharose beads and separated by SDS-PAGE, followed by immunoblotting with specific antibodies for Cdc42.

Cell morphological observation. MHCC97-L cells ($5x10^4$) were transiently transfected for 24 h with the pEGFP-N1 or pEGFP-BNIPL-2 plasmid, and then they were loaded onto
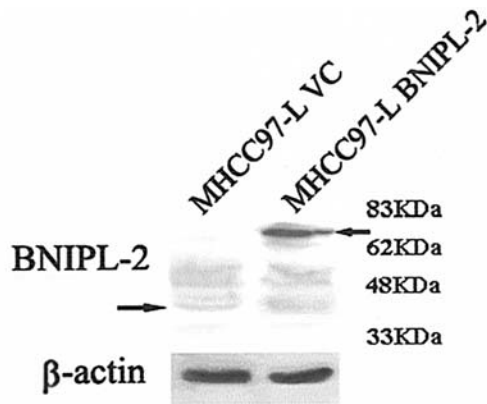
Figure 1. Immunoblot analysis of BNIPL-2 expression. Equal amounts of total cell protein were analyzed using the BNIPL-2 antibody. Positions of the molecular weight markers are indicated on the right. ß-actin is shown as the loading control.

glass coverslips. After incubation at 37°C/5% CO_2, cell morphology was viewed with a microscope by direct fluorescence detection and representative pictures were taken.

Statistical analysis. All the experiments were performed in triplicates as indicated. Fisher's exact test and the Student's t-test were used, respectively, for comparisons of the enumeration data (number of mice with metastases) and other measurement data. $P < 0.05$ was considered as statistically significant.

Results

Overexpression of BNIPL-2 in MHCC97-L cells. MHCC97-L cells were transfected with the pEGFP-BNIPL-2 or pEGFP-N1 vector. The expression of BNIPL-2 in the transfectants was analyzed by immunoblotting using the BNIPL-2 antibody (Fig. 1). In the MHCC97-L VC cells, the band of endogenous BNIPL-2 expression migrated at the expected position (~43 kDa), which was almost invisible. The major band of remarkably overexpressed BNIPL-2 migrated at ~70 kDa in the MHCC97-L BNIPL-2 cells.

Cell invasion and migration of MHCC97-L BNIPL-2 cells. In the cell invasion assays, the MHCC97-L cells were found to exhibit a pronounced cell invasion. The MHCC97-L BNIPL-2 cells that migrated to the lower surface of the filter were five times more than the MHCC97-L VC cells (Fig. 2A). Wound healing assay was then used as a measure of the rate of cell migration or movement (12). The data indicate that the MHCC97-L BNIPL-2 cells migrating into the wound were about seven times as many as the MHCC97-L VC cells (Fig. 2B). These results demonstrate that the overexpression of BNIPL-2 in MHCC97-L cells promotes cell invasion and migration *in vitro* ($P < 0.05$).

BNIPL-2 promotes the metastasis of HCC cells in vivo. The xenografts were implanted into the livers of two nude mice groups. When the animals were sacrificed and autopsied, we found that the BNIPL-2 protein was still overexpressed in the xenografts of the MHCC97-L BNIPL-2 group (Fig. 3A). Four
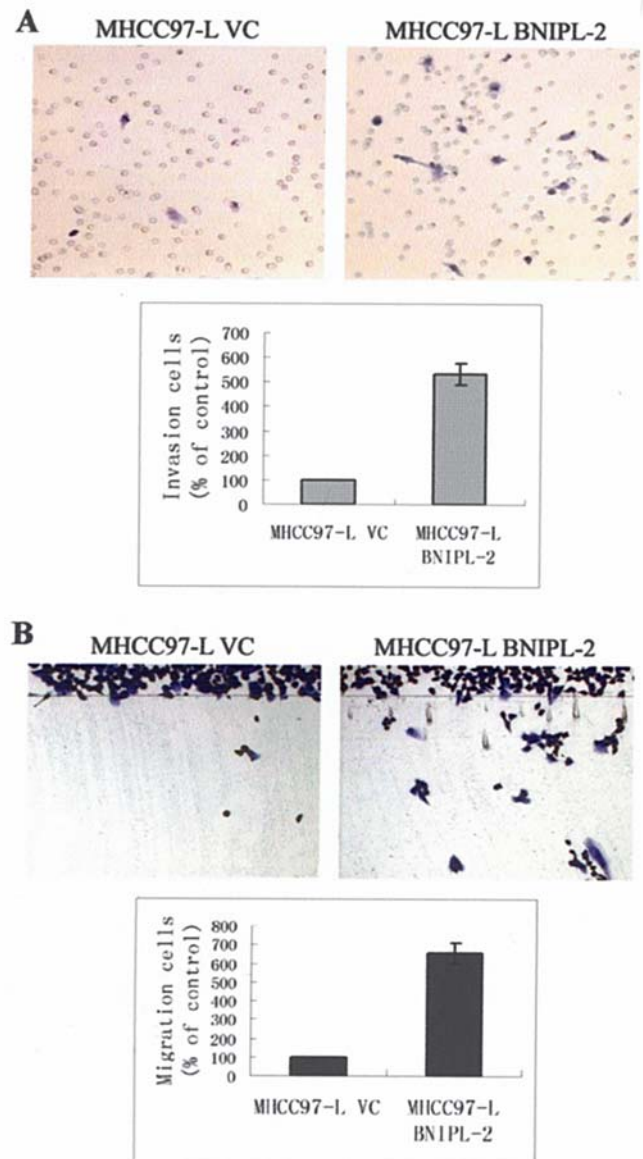

Figure 2. BNIPL-2 promoted invasion and migration *in vitro*. The results are the average of three independent experiments with five fields counted per experiment. Bars represent the SE. Data are expressed as the means ± SE from three replicate experiments ($P < 0.05$). (A) Matrigel invasion assay indicated an increase in cell invasion (x100 magnification). The fields show the attached cells that successfully reached out and invaded the other side of the Matrigel membrane. Cells passing through the Matrigel membrane were found to increase significantly when BNIPL-2 was overexpressed. (B) The wound-healing assay shows the effects of BNIPL-2 on the motility of human hepatocellular carcinoma cells with low metastatic potential (MHCC97-L) (x100 magnification). A significant acceleration in cell motility was revealed in the MHCC97-L BNIPL-2 cells compared with the control.

of 7 mice in the MHCC97-L BNIPL-2 group showed intrahepatic metastasis (Fig. 3B). The metastatic ratio of intrahepatic metastasis was obviously increased compared with the MHCC97-L VC group (0/7, 0%), as shown in Table I. Furthermore, pulmonary metastasis was observed in the lung tissue sections (Fig. 3C) in the MHCC97-L BNIPL-2 group (7/7, 100%), much more than the ratio of pulmonary metastasis in the MHCC97-L VC group (2/7, 28.6%, Table I). These results indicate that the overexpression of BNIPL-2 enhanced the metastatic ability of the HCC cells *in vivo*.
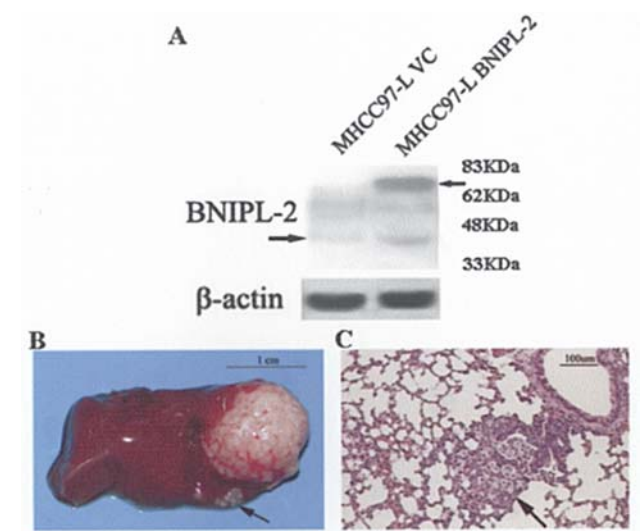
Figure 3. BNIPL-2 enhanced cancer metastasis *in vivo*. (A) BNIPL-2 expression in the xenografts. Lysates from the xenografts were immuno-blotted for detection of the BNIPL-2 expression. Positions of the molecular weight markers are indicated on the right. ß-actin is shown as the loading control. (B) Intrahepatic metastasis in the xenografts. The black arrow represents the metastatic nodules. (C) Pulmonary metastasis in the xenograft models. The black arrow represents the metastatic human HCC cells in the lung tissues of the mice. H&E staining.

Table I. Comparison of the metastatic ratio between the MHCC97-L VC and MHCC97-L BNIPL-2 group.

	MHCC97-L VC	MHCC97-L BNIPL-2	P-value
Intrahepatic	0/7, 0%	4/7, 57.1%	0.035
Pulmonary	2/7, 28.6%	7/7, 100%	0.010

P indicates the possibility using Fisher's exact test

Cdc42 activition and filopodia formation. The GST-PAK-CD protein was used to pull down the activated Cdc42, Cdc42-GTP, from the extracts of the cells. As shown in Fig. 4A, the basal activity of Cdc42 was low in the MHCC97-L VC cells; and the MHCC97-L BNIPL-2 cells had an obvious increase in the level of Cdc42-GTP.

With direct fluorescence microscopy, we found that the MHCC97-L cells transiently transfected with the pEGFP-N1 plasmid were mostly round in shape and <5% of them had some background with irregular shapes including filopodia. However, when the plasmid DNAs of pEGFP-BNIPL-2 were transiently transfected into the MHCC97-L cells, the cells were more irregular in shape. BNIPL-2 induced filopodia formation in almost 50% of the transfected cells (Fig. 4B). The data reveal that BNIPL-2 caused the increase in Cdc42 activity and the formation of filopodia, which led to cell migration and cancer metastasis.

Upregulation of CD44 in MHCC97-L BNIPL-2 cells. Using Western blotting, we examined the expression of the cell
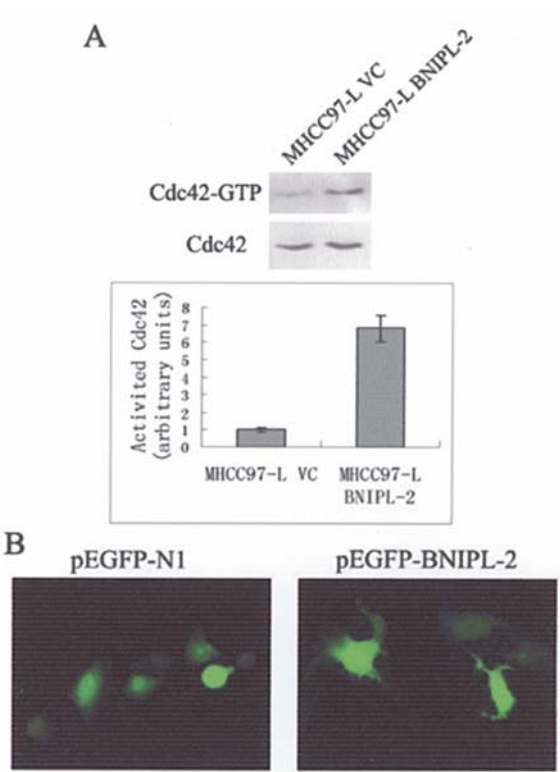

Figure 4. Cdc42 GTPase activation and filopodia formation. (A) The activation of Cdc42. The top panel shows the active Cdc42 in the GTP-bound form; the lower panel indicates the total amount of Cdc42 in 10 μg total cell extract. The intensity of the bands corresponding to Cdc42-GTP was quantified according to the amount of total Cdc42. The results are shown by the means ± SE of three independent experiments (P<0.05). (B) BNIPL-2 induced filopodia formation. The MHCC97-L cells transiently transfected for 24 h with the GFP expression plasmids pEGFP-BNIPL-2 or the control vector pEGFP-N1, were visualized for cell morphology by direct fluorescence detection.
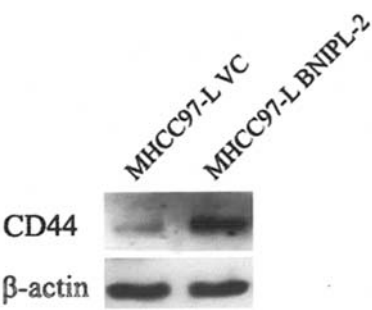

Figure 5. The effects of BNIPL-2 on CD44 expression in cancer cells. CD44 was highly expressed in the MHCC97-L BNIPL-2 cells. Lysates from the cell lines were immunoblotted in order to examine the expression of CD44. ß-actin is shown as the loading control.

surface adhesion molecule, CD44, which was involved in cell migration and invasion. Our results show that the over-expression of BNIPL-2 in the MHCC97-L cells led to the upregulation of the protein expression of CD44 (Fig. 5).

Discussion

HCC is one of the most common and aggressive human malignancies (13). Primary HCC can be partly treated with

either surgery or chemotherapy. However, a major complication of such treatment regimens results from the development of metastases, the distant tumor colonies derived from the spread of cancer cells from a primary tumor through the blood or lymphatic system (14). Despite remarkable advances in diagnostic and therapeutic techniques for cancer, the prognosis of HCC patients remains poor due to metastasis.

The overexpression or deregulation of the GTPase or some element of the Rho pathway has been reported in many human cancers (15). Rho-family GTPases are a multimember family of proteins involved in diverse cellular functions that are related to cell growth, development, apoptosis, tumorigenesis, and metastasis (16). Our previous assays of protein-protein interactions have shown that BNIPL-2 interacted with Cdc42GAP through the BCH domain locateed at the C-terminus and suppressed the Cdc42GAP activity towards Cdc42 by adding BNIPL-2 into the *in vitro* GAP activity assay system (5). However, it is still unclear whether BNIPL-2 is involved in the process of cancer metastasis via the Cdc42 pathway.

Since the signal pathways triggered by Cdc42 modulate distinct cytoskeletal rearrangement and the formation of filopodia for cell motility, we wanted to elucidate whether BNIPL-2 affects the morphology and migration potentials of the target cells via the Cdc42 pathway. In this report, our data indicate that BNIPL-2 not only enhanced the migration and invasion of cancer cells *in vitro* (Fig. 2), but also promoted cancer metastasis *in vivo* (Fig. 3, Table I), which was consistent with the result that BNIPL-2 was involved in the activition of the small GTPase, Cdc42, and the promotion in filopodia formation (Fig. 4).

We also selected another stable cell line expressing the BNIPL-2 protein in the same conditions and repeated the metastatic assays *in vitr*o and *in vivo*. Enhanced cell migration and cancer metastasis were also observed, although the level of BNIPL-2 overexpression in the second stable cells was almost 20% as much as in the MHCC97-L BNIPL-2 cells (data not shown).

CD44 is a transmembrane receptor for the extracellular matrix (ECM) components including hyaluronic acid (HA) (17) and is implicated in a wide variety of adhesion-dependent cellular processes including lymphocyte homing (18), cell migration (19,20), and tumor cell metastasis and invasion (21-23). The activation of the Rho family of small G proteins, Cdc42, is responsible for the Ha-RasVal-12-induced CD44 cleavage (24,25). CD44 expressed in cancer cells is proteolytically cleaved through membrane-anchored metalloproteases and CD44 cleavage plays a critical role in cancer cell migration (25,26). Therefore, we performed Western blot analysis with the antibody against CD44 and found that CD44 expression waas upregulated when BNIPL-2 was overexpressed in MHCC97-L cells (Fig. 5), which facilitated cancer cell migration and invasion. However, the molecular pathway of CD44 expression upregulated by BNIPL-2 remains to be elucidated.

As cancer metastasis is a complicated process, many molecular characteristics are still unknown. In the present study, we firstly provided evidence that BNIPL-2 increases the invasion of cancer cells *in vitro* and promotes the metastasis of human HCC *in vivo*. Furthermore, it is the potential molecular mechanism that BNIPL-2 enhances cancer metastasis via maintaining the high level of the active Cdc42 form, Cdc42-GTP, and upregulating the expression of CD44. These results link BNIPL-2 with cancer metastasis and allow us to further analyze its signaling pathway involved in the invasion and metastasis of cancer.

Acknowledgements

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