β-inducible gene-h3 promotes human breast carcinoma cell metastasis by activating the phosphatidylinositol 3-kinase/protein kinase B signaling pathway

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Abstract. Metastatic breast cancer is one of the most common metastatic tumors. Although studies have validated the role of β-inducible gene-h3 (βig-h3) in human biology and disease, the detailed mechanisms mediated by βig-h3 in breast carcinoma metastasis remain unclear. Thus, the present study investigated the role and potential mechanism of βig-h3 during breast carcinoma cell metastasis. The results indicated that the upregulation of βig-h3 significantly promotes the growth and inhibits the cisplatin-induced apoptosis of breast carcinoma cells. It was also demonstrated that βig-h3 promoted the migration and invasion of human breast carcinoma cells *in vitro* and *in vivo*. Furthermore, the results demonstrated that βig-h3 upregulated the overall expression and phosphorylation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) in human breast carcinoma cells. By contrast, βig-h3 knockdown reversed the βig-h3-mediated characteristics of breast carcinoma cells. Thus, the current study demonstrated that the PI3K/Akt signaling pathway serves a role in βig-h3-induced human breast cancer cell metastasis and that βig-h3 transfection enhances the metastatic potential of human breast carcinoma cells via the PI3K/Akt signaling pathway. These observations contribute to the understanding of the potential mechanism of human breast carcinoma cell growth and metastasis and suggest that βig-h3 may be a promising therapeutic target for the treatment of human breast carcinoma.

Introduction

Metastatic breast cancer is one of the most common types of metastatic tumors and affects the health of women around the world (1). The mortality rate of patients with breast carcinoma is high due to local invasion and distant metastasis and >30% of patients with breast carcinoma develop metastasis during progression of their disease (2,3). The 5-year overall survival rate of patients with breast cancer is 4-28% and reports have indicated that the incidence of breast cancer in young women is growing and is frequently metastatic following diagnosis (4,5). Therefore, it is critical to analyze the potential mechanism(s) associated with local invasion and distant metastasis to improve the 5-year overall survival rate and prognosis of patients with breast carcinoma (6,7).

Transforming growth factor (TGF)-β-inducible gene-h3 (βig-h3) is highly expressed in various types of tumors in humans and is associated with the growth and metastasis of tumor cells (8-10). βig-h3 encodes a secreted extracellular matrix (ECM) protein and it has been demonstrated that this protein is induced by TGF-β in pancreatic cancer cells, subsequently stimulating the growth and invasion of pancreatic cancer cells (11). Furthermore, previous studies have indicated that the ECM protein TGF-βig-h3 promotes colon, gastric and ovarian cancer metastasis by enhancing cell extravasation (12-14).

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway is associated with cancer cell growth and metastasis and serves a role in the development of chemoresistance to platinum-based neoadjuvant chemotherapy (15). In a previous study, measurement of the cellular and molecular responses to lapatinib and Akt inhibitors suggested that they suppress breast cancer growth and aggressiveness (16). In addition, inhibiting PI3K/Akt downregulates the breast cancer resistance protein resensitized MCF7 breast cancer cell line, which promotes the apoptosis of MCF7 cells induced by mitoxantrone chemotherapy (17). Furthermore, a previous study indicated that the binding of matrix metalloproteinase-9-degraded fibronectin to integrin promotes the invasion of breast cancer cells via the focal adhesion kinase-Src-associated extracellular signal-regulated kinase 1/2 and PI3K/Akt/Smad-1/5/8 pathways (18). These results indicate that inhibiting the PI3K/Akt signaling pathway may suppress the growth of breast cancer cells.

The present study investigated the role of βig-h3 in the progression of breast carcinoma *in vitro* and *in vivo*. The

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potential mechanism of βig-h3-mediated growth and aggressiveness of breast carcinoma cells was also investigated. The results indicated that βig-h3 promotes the growth and metastasis of breast carcinoma cells. This may increase understanding of the signaling pathways that are activated during the metastasis of breast cancer cells and indicate that βig-h3 may be a potential therapeutic target to treat breast cancer.

Materials and methods

Cell culture. The breast cancer cell lines MCF-7, BT474 and SKBR3 and the normal breast cell line MCF-10A were purchased from the American Type Culture Collection (Manassas, VA, USA). All tumor cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 3 mM L-glutamine, 50 μg/ml gentamicin (Biowhittaker Reagents; Lonza Group, Ltd., Basel, Switzerland) and 1% penicillin/streptomycin. MCF-10A cells were cultured in a mammary epithelial cell medium (cat. no. 7611, MEpiCM; ScienCell Research Laboratories, Inc., San Diego, CA, USA). MCF-7 is the typical human breast carcinoma cell, therefore, this cell line was chosen for further experiments. All cells were cultured at 37˚C in 5% CO_2.

Transfection of small interfering RNA (siRNA). A total of $1x10^8$ cells (MCF-7, BT474, SKBR3 and MCF-10A) were transfected with either si-Rβig-h3 for knockdown of CHOP (Kd-CHOP) or si-Rvector (Invitrogen; Thermo Fisher Scientific, Inc.). MCF-7 cells ($1x10^6$) were transfected with 120 pmol si-Rβig-h3 (5'-CCUUUACGAGAC CCUGGGATT-3' and 5'-UCCCAGGGUCUCGUAAAG GTT-3') targeting βig-h3 or si-R vector as a control (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a Cell Line Nucleofector kit L (Lonza Group, Ltd.). Following 48 h transfection, cells were used for further analysis.

Overexpression of PI3K or βig-h3. MCF-7 ($1x10^6$) cells were cultured until 85% confluence was reached; media was then removed and cells were washed three times with PBS. MCF-7 cells were then transfected with pLentivirus-PI3K (pPI3K), plentivirus-βig-h3 (pβig-h3, 100 pmol) or plentivirus-vector (pVector, 100 pmol) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific., Inc.) according to manufacturer's instructions. Following 72 h transfection, cells were used for further analysis.

MTT assay. PI3K- and βig-h3-overexpressed, pVector-transfected, si-Rβig-h3-treated or si-Rvector-treated MCF-7 cells ($1x10^3$ per well) were cultured in 96-well plates for 48 h at 37˚C in triplicate for each condition and pVector-transfected MCF-7 was used as a control. Following incubation, 20 μl MTT (5 mg/ml) in PBS solution was added and dissolved in dimethyl sulfoxide for a further 2 h at 37˚C. Optical density was measured using an ELISA reader at a wavelength of 450 nm.

Apoptosis assay. βig-h3-overexpressed, pVector-transfected, si-Rβig-h3-treated or si-Rvector-treated MCF-7 cells were grown at 37˚C with 5% CO_2 until 90% confluence was reached. Cells were then incubated with cisplatin (2.0 mg/ml) or PBS for 48 h at 37˚C, trypsinized and collected. Cells were then washed in cold PBS, adjusted to $1x10^6$ cells/ml with PBS, labeled with Annexin V-fluoroscein isothiocyanate and propidium iodide (Annexin V-fluoroscein isothiocyanate kit; BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at 37˚C and analyzed using a FACScan flow cytometer (BD Biosciences) to assess apoptosis. Treatments were performed in triplicate and the percentage of labeled cells undergoing apoptosis in each group was determined and calculated using a Coulter EPICS XL Flow Cytometer and the results were analyzed using Expo32-ADC v. 1.2B software (Beckman Coulter Inc., Brea, CA, USA).

Cell migration and invasion assays. Sable PI3K-overexpressed, βig-h-overexpressed, pVector-transfected, si-Rβig-h3-treated or si-Rvector-treated MCF-7 cells were cultured in serum-free medium for 72 h at 37˚C. Migration and invasion assays were conducted in a 6-well culture plate with Transwell inserts (BD Biosciences). A total of $1x10^4$ MCF-7 cells/well were placed into the upper chamber in DMEM supplemented with 5% FBS for migration assays. For invasion assays, MCF-7 cells ($1x10^4$/well) were placed into the upper chamber with a Matrigel-coated membrane in DMEM supplement with 5% FBS for 72 h at 37˚C. The cells that invaded through the membrane were fixed with 3% formaldehyde for 15 min at 37˚C and stained with 0.5% crystal violet for 10 min at 37˚C. The invasion and migration of tumor cells were assessed in a minimum of three randomly selected fields using an inverted microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Western blot analysis. MCF-7 cells were harvested by scraping and were subsequently lysed in radioimmunoprecipitation buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) followed by homogenization at 4˚C for 10 min. Protein concentration was measured by a BCA protein assay kit (Thermo Scientific, Pittsburgh PA, USA). Protein (20 μg) was analyzed using 12% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer (5% milk) for 2 h at 37˚C prior to incubation with primary antibodies at 4˚C overnight. Proteins were incubated with rabbit anti-human βig-h3 (cat no. ab170874, 1:1,000 dilution, Abcam, Cambridge, MA, USA), PI3K (cat no. ab86714, 1:1,000 dilution, Abcam), pAKT (cat no. ab81283, 1:1,000 dilution, Abcam), Akt (cat no. ab8805, 1:1,000 dilution, Abcam) and β-actin (1:2,000 dilution, cat no. ab5694; Abcam, Cambridge, UK) antibodies for 12 h at 4˚C. Proteins were the incubated with horseradish peroxide (HRP)-labeled secondary goat anti-rabbit antibodies (1:5,000 dilution, ab205718, Abcam) for 2 h at 37˚C and protein expression was analyzed using a chemiluminescence detection system (Version 3.0, Sigma-Aldrich, Merck KGaA). The density of the bands was analyzed by Quantity One software (version 4.62, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Animal study. A total of 30 specific pathogen-free (SPF) female nude Balb/c mice (6-8 weeks old) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All rats were housed in a temperature-controlled facility at

23±1˚C with a relative humidity of 50±5% and were exposed to a 12-h light/dark cycle. The mammary glands of mice were subcutaneously implanted with βig-h3-overexpressed, si-Rβig-h3-treated or si-Rvector-transfected MCF-7 cells ($1x10^7$) and were subsequently divided into three groups (n=10 in each group). Mice were observed for 40 days following tumor inoculation. Tumor diameters were recorded every 2 days and tumor volume was calculated using the following formula: 0.52 x smallest diameter^2 x largest diameter (17). Experimental mice were euthanized when the tumor diameter reached 10 mm. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China (18). The present study was also approved by the Ethics Committee of Pingdu People's Hospital (Qingdao, China). All surgery was performed following intraperitoneal injection of sodium pentobarbital (40 mg/kg, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to induce anesthesia and all efforts were made to minimize suffering.

Tumor metastasis assay. Animals were euthanized by overdose with pentobarbital (intraperitoneal, 120 mg/kg) on day 40. Mice were dissected and the distribution of the tumor mass was observed in the lung, liver, bowel, bone, brain and pleura. The tumor metastasis rate was recorded in each group (n=6) according to the numbers of tumors as described previously (19).

Immunohistochemistry. Tumor tissues from xenografted mice were fixed using 10% formaldehyde for 30 min at 37˚C, washed with PBS and followed with embedding in paraffin wax. Tissues were deparaffinized in xylene and rehydrated in grade alcohols. They were then cut into 4-μm thick serial sections. Antigen retrieval was then performed on tumor sections using Antigen Retrieval Reagents (cat. no. #CTS015, Bio-Rad Laboratories, Inc.). Tumor sections were blocked with 5% milk for 2 h at 37˚C and then incubated with goat anti-human βig-h3 (cat no. ab170874, 1:1,000 dilution), PI3K (1:1,000 dilution, cat no. ab86714) pAKT (cat no. ab81283, 1:1,000 dilution) or Akt (1:1,000 dilution, cat no. ab8805; all Abcam) antibody for 12 h at 37˚C. Sections were then incubated with HRP-labeled secondary goat anti-rabbit antibodies (1:2,000 dilution, ab150077, Abcam) and visualized using ZEISS LSM 510 confocal microscope at a magnification of x40.

Statistical analysis. All data are expressed as the mean ± standard deviation of triplicate dependent experiments. The results were analyzed using Student's t test or one-way analysis of variance followed by a Tukey's honest significant difference test. All data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) and Graphpad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA), as well as Microsoft Excel (version 2010, Microsoft Corporation, Redmond, WA, USA). $P<0.05$ was determined to indicate statistically significant difference.

Results

Effects of βig-h3 on breast carcinoma cell growth. The expression of βig-h3 was analyzed in the breast cancer cell lines MCF-7, BT474 and SKBR3 and in the normal breast cell line MCF-10A, which acted as the control. The expression of βig-h3 was significantly increased in MCF-7, BT474 and SKBR3 cells compared with MCF-10A cells (Fig. 1A). It was also demonstrated that pβig-h3 promoted βig-h3 expression, while si-Rβig-h3 decreased βig-h3 expression in MCF-7, BT474 and SKBR3 cells (Fig. 1B). The results also demonstrated that βig-h3 transfection significantly promoted the growth of MCF-7, BT474, SKBR3 and MCF-10A cells compared with their vector transfection controls (Fig. 1C). However, knockdown of βig-h3 expression by si-Rβig-h3 significantly inhibited the growth of MCF-7, BT474 and SKBR3 cells compared with the controls transfected with si-R vector (Fig. 1D). These results suggest that βig-h3 promotes the growth of breast carcinoma cells.

Transfection of βig-h3 promotes the migration and invasion of breast carcinoma cells. The effects of βig-h3 on the migration and invasion of breast carcinoma cells were investigated in MCF-7 cells following transfection. The results demonstrated that the migration and invasion ability of MCF-7 cells was significantly increased in cells transfected with pβig-h3 compared with control cells (Fig. 2A and B). By contrast, knockdown of βig-h3 by si-Rβig-h3 significantly inhibited the migration and invasion of MCF-7 cells (Fig. 2C and D). These results suggest that βig-h3 promotes the migration and invasion of breast carcinoma cells.

Transfection of βig-h3 inhibits the apoptosis of breast carcinoma cells induced by cisplatin. Following transfection, the apoptosis of breast carcinoma cells was analyzed by incubation with cisplatin. The apoptosis rate of MCF-7 cells was significantly suppressed in cells transfected with pβig-h3 compared with the control (Fig. 3A). By contrast, knockdown of βig-h3 by si-Rβig-h3 significantly promoted the apoptosis of MCF-7 cells induced by cisplatin compared with the control (Fig. 3B). These results indicate that βig-h3 expression is associated with the apoptosis of breast carcinoma cells induced by cisplatin.

Knockdown of βig-h3 inhibits the growth and aggressiveness of breast carcinoma cells via the PI3K/Akt signaling pathway. It was subsequently determined whether βig-h3 promotes the growth, migration and invasion of breast carcinoma cells via the PI3K/Akt signaling pathway. The results indicated that the expression of PI3K and Akt was significantly increased in MCF-7 cells transfected with pβig-h3 compared with controls (Fig. 4A). By contrast, the expression of PI3K and Akt was significantly decreased in MCF-7 cells transfected with si-Rβig-h3 compared with the control (Fig. 4B). The results also demonstrated that PI3K upregulation reversed the effects of si-Rβig-h3 on the expression of Akt in MCF-7 cells (Fig. 4C). In addition, PI3K upregulation reversed the effects of si-Rβig-h3 on the growth, migration and invasion of MCF-7 cells (Fig. 4D-F). These data suggest that βig-h3 promotes the growth and metastasis of breast carcinoma cells via the PI3K/Akt signaling pathway.

Knockdown of βig-h3 suppresses the formation of breast tumor masses in xenografted mice. The role of βig-h3 in the formation of breast carcinoma was investigated by implanting βig-h3-upregulated or βig-h3-knockdown MCF-7 cells into

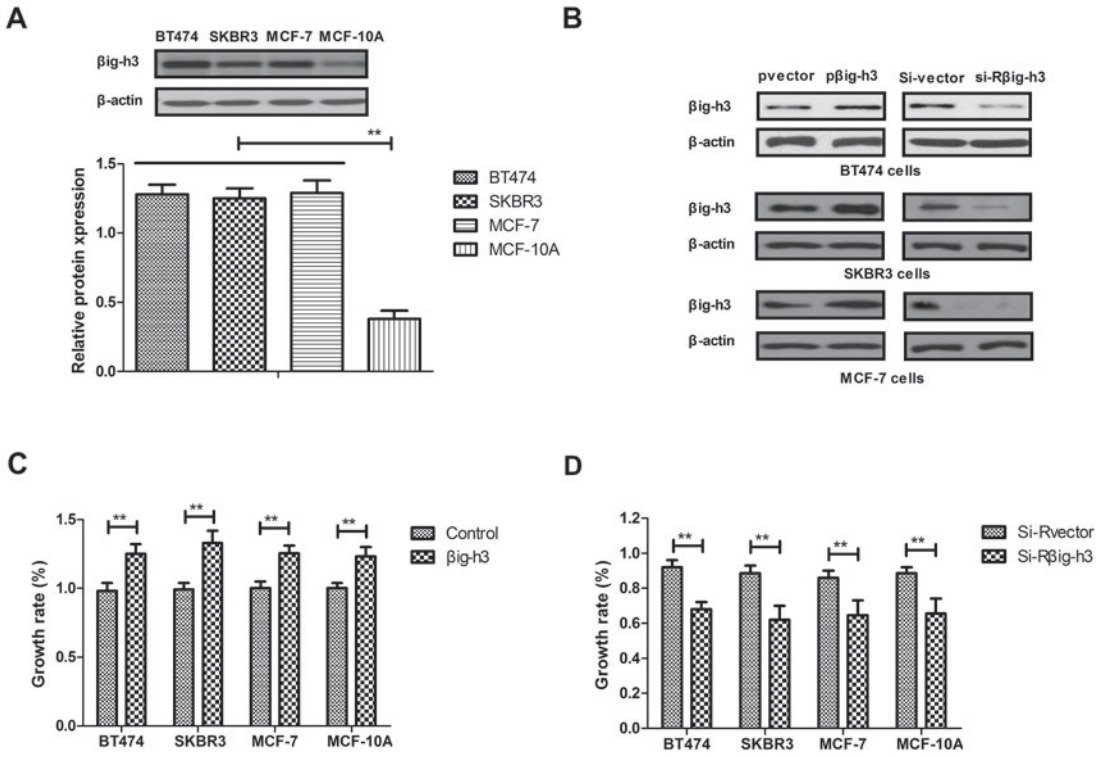

Figure 1. βig-h3 promotes the growth of breast carcinoma cells. (A) βig-h3 expression in the breast cancer cell lines MCF-7, BT474, SKBR3 and the normal breast cell line MCF-10A was determined using western blotting. (B) The expression of βig-h3 in MCF-7, BT474 and SKBR3 cells transfected with pvector, p βig-h3, si-vector or si-Rβig-h3 was determined using western blotting. (C) The effect of βig-h3 upregulation on MCF-7, BT474 and SKBR3 cell growth was determined using an MTT assay. (D) Effect of βig-h3 knockdown on MCF-7, BT474 and SKBR3 cell growth was determined using an MTT assay. Values are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. **P<0.01. βig-h3, β-inducible gene-h3; si-R, small interfering RNA.

experimental mice. The results demonstrated that tumor weight in the si-Rβig-h3 group was significantly decreased compared with the control and pβig-h3 groups, suggesting that βig-h3 knockdown inhibits the formation of breast tumor masses. By contrast, tumor weight in the pβig-h3 group was significantly increased compared with the control, indicating that βig-h3 upregulation promotes the growth of breast tumors (Fig. 5A). It was also determined that the metastasis rate was significantly decreased in the si-Rβig-h3 group compared with the control and pβig-h3 groups, suggesting that βig-h3 knockdown inhibits breast carcinoma metastasis compared with the control (Fig. 5B). By contrast, βig-h3-upregulation significantly promoted breast carcinoma metastasis in the subcutaneous tissue of experimental mice, suggesting that βig-h3 upregulation stimulates carcinoma metastasis. Immunohistochemistry analysis indicated that the expression of βig-h3, PI3K and Akt were markedly increased in βig-h3-overexpressed breast tumors, while PI3K and Akt expression were markedly decreased in MCF-7 breast tumors following βig-h3 knockdown (Fig. 5C). The expression of phosphorylated Akt was also downregulated following βig-h3-knockdown in MCF-7 breast tumors (Fig. 5D). These results therefore suggest that βig-h3 knockdown suppresses the formation of breast tumor masses *in vivo*.

Discussion

The number of women that are diagnosed with breast cancer had increased since 2010, and the majority receive breast-conserving surgery followed by adjuvant radiotherapy, chemotherapy and/or comprehensive treatment (20). The results of previous studies have suggested that increased βig-h3 expression promotes migration and invasion in different types of human cancer cells, including intraductal carcinoma cells, breast mucinous adenocarcinoma cells and medullary breast carcinoma cells (8,12,13). However, the role of βig-h3 in the progression of human breast cancer remains unknown. The present study investigated the effects of βig-h3 on the growth, migration, invasion and apoptosis of human breast cancer cells. The association between the βig-h3-mediated PI3K/Akt signaling pathway, and cell growth, invasion and migration in human breast cancer cells was also assessed. The results suggested that βig-h3 upregulation promotes the growth and invasion of breast cancer cells, whereas βig-h3 knockdown inhibits the growth and invasion of breast cancer cells via downregulation of the PI3K/Akt signaling pathway, which also decreases the resistance of breast carcinoma cells to apoptosis following its induction by cisplatin.

βig-h3 is a protein that serves a role in the proliferation, migration, apoptosis and differentiation of tumor cells (14). It has been suggested that βig-h3 promotes carcinogenesis in different types of cancer (21). In addition, Kim *et al* (22) demonstrated that βig-h3 interacts with α3β1 integrin to promote the adhesion and migration of human hepatoma cells by activating focal adhesion kinase-paxillin signaling. Furthermore, βig-h3 promotes the adhesion, migration and proliferation of gastric cancer cells in peritoneal

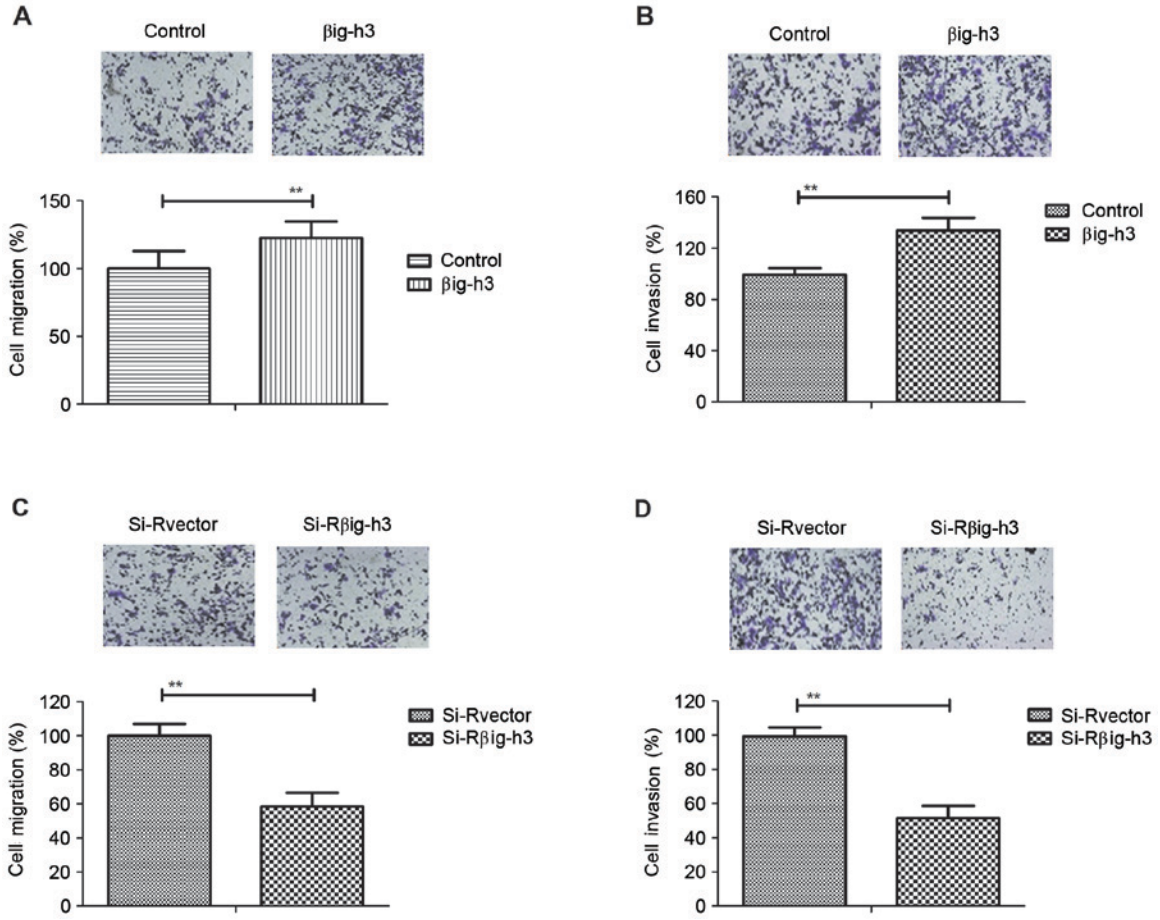

Figure 2. Upregulation of βig-h3 promotes the migration and invasion of breast carcinoma cells. Upregulation of βig-h3 significantly promotes the (A) migration and (B) invasion of MCF-7 cells compared with the control. Knockdown of βig-h3 significantly inhibits MCF-7 cell (C) migration and (D) invasion following 72 h incubation *in vitro* compared with the control. Magnification, x40. Values are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. **P<0.01. βig-h3, β-inducible gene-h3; si-R, small interfering RNA.

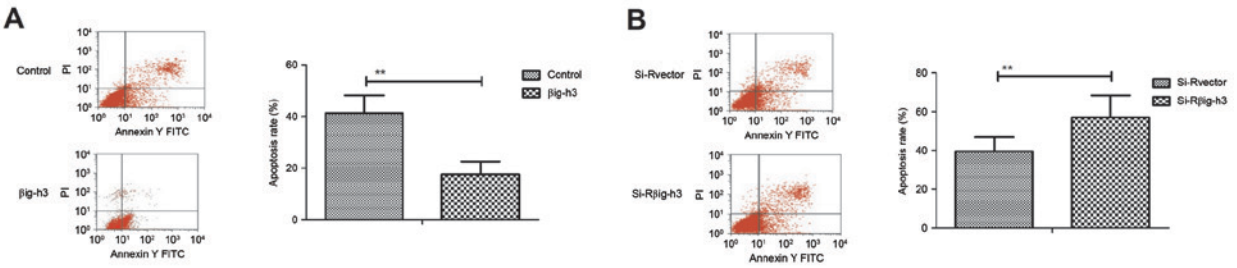

Figure 3. Transfection of βig-h3 suppresses the apoptosis of breast carcinoma cells induced by cisplatin. (A) Upregulation of βig-h3 decreases the apoptotic rate of MCF-7 cells compared with the control following 48 h incubation, as determined using flow cytometry. (B) βig-h3 knockdown promotes the apoptosis of MCF-7 cells compared with the control following 48 h incubation, as determined using flow cytometry. Values are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. **P<0.01. βig-h3, β-inducible gene-h3; si-R, small interfering RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide.

carcinomatosis (8,22). Jeong and Kim (23) demonstrated that transforming growth factor-β1 enhances βig-h3-mediated gastrointestinal tract tumorigenesis migration via the FAK/Akt/Akt1S1/PRS6/EIF4EBP pathways. The results of the present study indicated that βig-h3 knockdown inhibited the growth, migration and invasion of breast carcinoma cells via the PI3K/Akt signaling pathway. In addition, it was indicated that the upregulation of βig-h3 promotes the growth, migration and invasion of breast cancer cells and also increases apoptotic resistance in breast cancer cells.

In conclusion, the results of the current study indicate that βig-h3 is associated with the growth and metastasis of breast cancer cells. The data suggest that the inhibition of PI3K and Akt expression induced by βig-h3 is associated with the growth, apoptosis and metastasis of breast cancer cells. Additionally, βig-h3 knockdown inhibits the proliferation and increases the apoptosis of breast carcinoma cells. These results imply that the decreased expression of βig-h3 decreases the risk of metastasis in patients with breast cancer, whereas the increased expression of βig-h3 may require

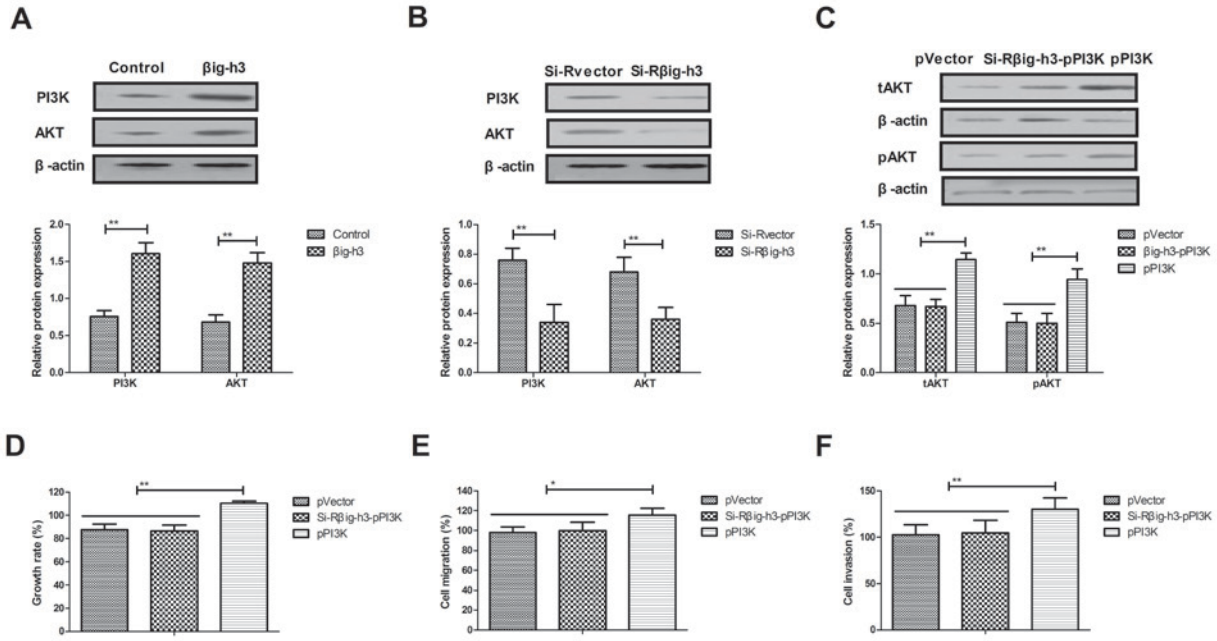

Figure 4. Transfection of βig-h3 regulates the growth, migration and invasion of breast carcinoma cells via the PI3K/Akt signaling pathway. (A) Upregulation of βig-h3 increases the expression of PI3K and Akt in MCF-7 cells compared with the control. (B) Knockdown of βig-h3 decreases PI3K and Akt expression in MCF-7 cells compared with the control following 72 h incubation. (C) PI3K upregulation reverses βig-h3-enhanced Akt expression and phosphorylation in MCF-7 cells. PI3K upregulation reverses the si-Rβig-h3-inhibited (D) growth, (E) migration and (F) invasion in MCF-7 cells. Values are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. *P<0.05, **P<0.01. βig-h3, β-inducible gene-h3; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; si-R, small interfering RNA; p, phosphorylated.

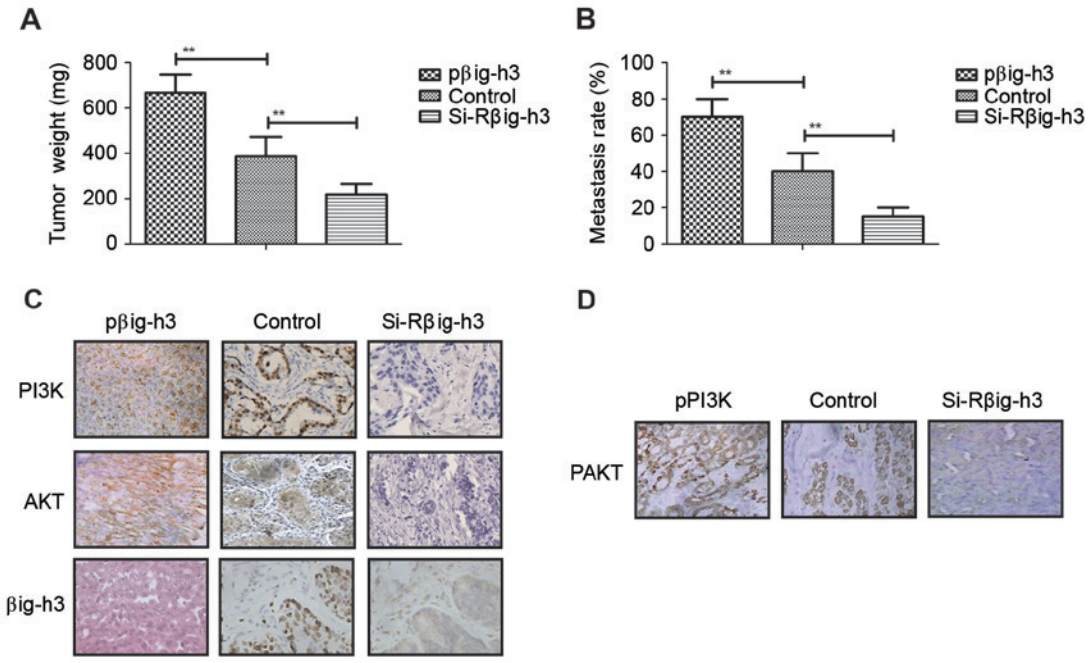

Figure 5. βig-h3 knockdown suppresses the formation of breast tumor mass and carcinoma metastasis in xenografted mice. (A) Tumor weight in the pβig-h3, control and si-Rβig-h3 groups. (B) The metastasis rate in the pβig-h3, control and si-Rβig-h3 groups. (C) Expression of βig-h3, PI3K and Akt in tumor tissues from the pβig-h3, control and si-Rβig-h3 groups, as determined by immunohistochemistry. (D) Expression of pAkt in tumor tissues from the pβig-h3, control and si-Rβig-h3 groups, as determined by immunohistochemistry. Magnification, x40. Values are presented as the mean ± standard error of the mean of three independent experiments performed in triplicate. **P<0.01. βig-h3, β-inducible gene-h3; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; si-R, small interfering RNA; p, phosphorylated.

the administration of intensive adjuvant chemotherapy to patients. It may therefore be advantageous to tailor therapy to individual patients, according to levels of βig-h3 expression, as higher βig-h3 expression seems to increase the risk of metastasis. The results indicate that βig-h3 is a potential molecular target for breast carcinoma treatment, which may contribute to understanding of the mechanism of human breast carcinoma metastasis.

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