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The oncoprotein HBXIP enhances migration of breast cancer cells through increasing filopodia formation involving MEKK2/ERK1/2/Capn4 signaling

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ABSTRACT

We have reported that the oncoprotein hepatitis B X-interacting protein (HBXIP) plays a crucial role in the promotion of migration of breast cancer cells. Lamellipodia and filopodia protrusions play fundamental roles, involving dynamic cytoskeleton reorganization in the metastasis of cancer. Here, we observed that the expression levels of both HBXIP and Calpain small subunit 1 (Capn4) were very high in clinical metastatic lymph nodes of breast tumor. Then, we found that HBXIP was able to up-regulate Capn4 at the levels of promoter, mRNA and protein in breast cancer cells through activation of ERK1/2. Moreover, we showed that HBXIP activated ERK1/2 through up-regulating MEKK2. In function, we revealed that HBXIP increased the filopodia formation through Capn4, resulting in cell migration. Thus, we conclude that the oncoprotein HBXIP enhances the migration of breast cancer through increasing filopodia formation involving MEKK2/ERK1/2/Capn4 signaling. Therapeutically, HBXIP may serve as a novel target in breast cancer.

Introduction

Cell motility plays a prominent role in a variety of biological processes, including embryonic development and tumor cell metastasis [1,2]. Cytoskeleton elements must be coordinately regulated in the cells to fulfill complex cellular functions. Cell migration is a cycle step from extending lamellipodia and filopodia to tractive cells, which is directly associated with the relocation of the concentration of cell cytoskeleton components [3]. Filopodia is a slender cytoplasmic projection that extends beyond the leading edge of membrane in migrating cells [4]. They contain actin filaments cross-linked into bundles by actin-binding proteins [5,6]. Filopodial retraction force is generated by cortical actin dynamics [7]. Therefore, actin displays a crucial role in the regulation of cytoskeletons.

Calpain small subunit 1 (Capn4) is a member of the Calpain family which plays a critical role in cell migration and rearrangement of actin cytoskeleton [8]. It has been reported that Capn4-deficient embryonic fibroblasts display reduced cell migration and disruption of the actin cytoskeleton [9]. Calpain activity is required for the stabilization of growth cone filopodia [10]. Capn4 overexpression underlies tumor invasion and metastasis after liver transplantation for hepatocellular carcinoma [11]. However, the regulatory mechanism of Capn4 in the migration of cancer cells is poorly understood.

Mammalian hepatitis B X-interacting protein (HBXIP) is a 18-kDa protein, which was originally identified by its interaction with the hepatitis B virus X protein and its sequence is well conserved among mammalian species [12]. HBXIP suppresses apoptosis in hepatoma cells, and regulates centrosome duplication in HeLa cells [13,14]. Our group reported that HBXIP also played crucial roles in the promotion of proliferation and migration of breast cancer cells through acting as a coactivator of transcription factors [15–18]. However, the mechanism by which HBXIP enhances migration of breast cancer cells is ill-documented.

In this study, we try to identify the mechanism by which HBXIP accelerates migration of breast cancer cells. Interestingly, we find that the oncoprotein HBXIP enhances the migration of breast cancer cell through MEKK2/ERK1/2/Capn4 signaling. Thus, our finding provides new insights into the mechanism of migration of breast cancer cells.

Abbreviations: HBXIP, hepatitis B X-interacting protein; Capn4, Calpain small subunit 1; MEKK2, mitogen-activated protein kinase kinase kinase 2; ERK1/2, extracellular signal-regulated kinase 1/2; IHC, immunohistochemistry; ChIP, chromatin immunoprecipitation.

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Materials and methods

Immunohistochemistry (IHC)

Breast cancer tissue array (No. 08C14), comprising duplicates of 49 cases of infiltrating primary carcinoma and 38 metastatic tumors, was purchased from Xi'an Aomei Biotechnology (Xi'an, China). Immunohistochemistry assay was performed as described previously [15]. The slides were incubated with anti-HBXIP or anti-Capn4 (Chemicon, Temecula, CA) antibody at 4 °C overnight. Rabbit polyclonal anti-HBXIP antibody was affinity-purified from the anti-serum prepared previously [19]. After incubation at room temperature for 30 min with biotinylated secondary antibody, the slides were incubated with streptavidin-peroxidase complex at room temperature for 30 min. Immunostaining was developed by using chromogen, 3,3′-diaminobenzidine (DAB), and counterstained with Mayer's hematoxylin.

Reverse transcription-PCR (RT-PCR) and real-time PCR

A total of 28 cases of breast cancer tissues were collected from patients undergoing resection of breast cancer in Tianjin First Center Hospital (Tianjin, China). The total RNA from cells was prepared with Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized by PrimeScript reverse transcriptase (TaKaRa Bio, Dalian, China) and oligo-(dT) following the manufacturer's instructions. Primers used to test HBXIP expression were reported previously [20]. Primers used in the study were as follows: GAPDH: sense, CATCACCATCTTC CAGGAGCG, GAPDH: anti-sense, TGACCTTGCCCACAGCCTTG; Capn4: sense, CCCCCACGC ACACATTA, Capn4: anti-sense, CGCTATCCATCACGGCCACCAT.

Cell culture, plasmids and siRNA

MCF-7 and LM-MCF-7 (a high metastasis subclone of MCF-7 cell line [18]) cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and 100 U/ml penicillin/streptomycin in humidified 5% CO_2 at 37 °C. Cells were pretreated for 1 h with 30 μM PD98059, or 10 μM U0126. Human Capn4 (GenBank™ accession number NM_001749.2) cDNA was PCR-amplified from cDNA reverse transcripted from total RNA extractive of MCF-7 cells. The primers we used were: Capn4: sense, CGGGGTACCATGTTCCTGGTTAACTCGTTC and Capn4: anti-sense, CCGCTCGAGTCAGGAATACATAGTCAGCTGC. The resulting products were cloned into the EcoRI/XhoI sites of pCMV-tag2B vector. The luciferase reporter vectors of Capn4 and MEKK2 promoters were used as in our previous study [21,22]. The siRNA used in this study were listed as follows: siControl: AAUGGUC AUGGUCUUAUUCC, siHBXIP: CGGAAGCGCAGUGAUGUU, siCapn4: GCUUUUG UUCUCAGUAC, siMEKK2: GAAUGAUGUCCGAGUCAAAdTdT. The siRNAs targeting ERK1/2, Raf-1, A-Raf, B-Raf and ERK5 were reported previously [23–25]. One day before transfection, cells were collected, and seeded into 6-well-culture-plate at 1×10^5 cells per well (n = 3, each group). Cells were transfected with corresponding plasmids or siRNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Luciferase reporter gene assays

The plasmids of pCMV-tag2B, pGL3-Capn4, pGL3-MEKK2 and renilla luciferase reporter vector pRL-TK were used as described previously [21]. Luciferase activities were normalized to *Renilla* luciferase activity. All the assays were performed in triplicate. Cells were seeded into 24-well plates at a density of 1×10^4 per well and cultured for 24 h at 60~70% confluence before transfection. Corresponding plasmids were transfected using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, USA). The luciferase reporter gene promoter activities of Capn4 or MEKK2 were determined 36 h post transfection using a dual-luciferase reporter gene assay kit (Promega, USA).

Western blot analysis

Western blotting was carried out by standard protocols. The antibodies used in this study were β-actin (Sigma), p-ERK1/2(phosphor-p44/p42 MAPK) (Cell Signaling Technology), ERK1/2 (p44/p42 MAPK) (Cell Signaling Technology), MEKK2 (BOSTER), ERK5 (Proteintech), A-Raf (Immunoway), B-Raf (Immunoway) and Raf-1 (Immunoway). All experiments were repeated 3 times.

Animal transplantation

All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications nos. 80-23, revised 1996) and were performed according to the institutional ethical guidelines for animal experiment. The MCF-7 or LM-MCF-7 cells transiently transfected with pCMV, pCMV-HBXIP, siControl or siHBXIP were subcutaneously injected into the flanks of 4-week-old male BALB/c athymic nude mice, respectively. After 26 days, the mice were sacrificed, and necropsies were performed.

Chromatin immunoprecipitation assays (ChIP)

The ChIP assay was performed using the EpiQuikTM Chromatin Immunoprecipitation Kit from Epigentek Group Inc. (Brooklyn, NY, USA). Protein–DNA complexes were immunoprecipitated with HBXIP antibodies or anti-RNA polymerase II as a positive control antibody, and with normal rabbit IgG as a negative control antibody. Amplification of soluble chromatin prior to immunoprecipitation was used as an input control. The primers were designed according to the reports of the promoters of the MAP3Ks [22,26]. Primers used in this study were: MEKK1: sense, CAAGACTATCTCCGTGAGAATCCG, MEKK1: anti-sense, CCCATCGCTGCGTTGTGAG; MEKK2: sense, TCCACCTGTTCATCCCTG, MEKK2: anti-sense, GAGCCAAGATTCCACCAC; MEKK3: sense, GAAACCGCCTTGGCTTCGC, MEKK3: anti-sense, CACCTCAGGAACGCT CCGAAA; negative control: sense, TCCACCT GTTCATCCCTG and negative control: anti-sense, GAGCCAAGATTCCACCAC.

Fluorescence staining

Cells were processed for indirect fluorescence staining as described previously [27]. FITC-phalloidin (Invitrogen, Carlsbad, CA, USA) was used to detect F-actin. Stained cells were observed with Nikon TE200 inverted microscope. Images were analyzed by Spot version 4.5 (Diagnostic Instruments Inc, MI, USA). The numbers of filopodia per cell and the length of filopodia in 20 cells in different visual fields were examined by using FiloDetect software [28].

Modified Boyden's chamber assays

Cells and transfected cells were processed by modified Boyden's chamber assays as described previously [27]. During the assay, 30 μM PD98059 was added into both the upper and lower wells at the same concentrations. The experiments were repeated 3 times.

Statistics

All data were presented as mean ± standard error of the mean and were analyzed by ANOVA or Student's *t* test using Prism 4.0 (GraphPad Software, CA). A p value of < 0.01 was considered statistically significant. All statistical tests were two-sided. Chi square distribution was used to test the percentage. Correlation between expression levels of HBXIP and Capn4 in tumorous tissues was explored using Pearson's correlation coefficient. Capn4 expression in primary breast carcinoma and adjacent normal breast tissues were compared using a Wilcoxon signed-rank test.

Results

The expression of HBXIP is positively associated with that of Capn4 in clinical metastatic lymph nodes and breast cancer tissues

It has been reported that Capn4 can facilitate the migration of cancer cells [11]. Recently, we have reported that the oncoprotein HBXIP is able to enhance the migration of breast cancer cells [15,29]. Therefore, we are interested in the relationship of expression between HBXIP and Capn4 in the event. Then, we examined the expression of HBXIP and Capn4 in clinical metastatic lymph nodes and breast cancer tissues by immunohistochemistry staining using tissue arrays from the same tissue paraffin block. Interestingly, our data showed that the positive rates of HBXIP and Capn4 were 94.7% (36/38) and 89.5% (34/38) in the metastatic lymph nodes tissue samples, respectively, which were higher than those of HBXIP (77.6%, 38/49) and Capn4 (73.4%, 36/49) in the primary tissue samples (chi square test, $p < 0.01$), supporting that HBXIP and Capn4 play important roles in the metastasis of breast cancer. Importantly, the positive rate of Capn4 was 82.4% (61/74) in the HBXIP-positive samples (Fig. 1A), suggesting that the expression of Capn4 is closely related to that of HBXIP in clinical breast cancer tissues. However, the positive incidence of Capn4 was only one in three HBXIP-positive samples in 13 breast normal tissues. In our previous study, we showed that the expression levels of HBXIP were obviously higher in breast cancer tumors than those in their adjacent normal tissues [15]. Here, we found that the mRNA levels of Capn4 were also higher in tumors than those in their adjacent normal tissues (n = 28) by qRT-PCR (Fig. 1B). Moreover, we concerned the correlation of expression between HBXIP and Capn4 in above tumor tissues. Our data showed that the expression levels of Capn4 had a