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ORIGINAL PAPER

High expression of FBP17 in invasive breast cancer cells promotes invadopodia formation

Prabhat Suman[1] · **Sarthak Mishra**[1] · **Harish Chander**[1]

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Abstract

Metastatic spread of the cancer is usually the consequence of the activation of signaling pathways that generate cell motility and tissue invasion. Metastasis involves the reorganization of cytoskeleton and cell shape for the swift movement of the cells through extracellular matrix. Previously, we have described the invasive and metastatic role played by one of the members (Toca-1) of CIP4 subfamily of F-BAR proteins. In the present study, we address the role of another member (FBP17) of same family in the invasion breast cancer cells. Here, we report that the formin-binding protein 17 (FBP17) is highly expressed at both mRNA and protein levels in breast cancer cells. The study showed the association of FBP17 with cytoskeletal actin regulatory proteins like dynamin and cortactin. To determine its role in extracellular matrix (ECM) degradation, we achieved stable knockdown of FBP17 in MDA-MB-231 cells. FBP17 knockdown cells showed a defect and were found to be compromised in the degradation of ECM indicating the role of FBP17 in the invasion of breast cancer cells. Our results suggest that FBP17 is highly expressed in breast cancer cells and facilitates the invasion of breast cancer cells.

Keywords Formin-binding protein 17 · Breast cancer · Invadopodia · Extracellular matrix

Introduction

The heterogeneity of tumor cells in breast cancer is a driving force for invasion and metastasis [1]. Metastasis is the leading cause of fatality in breast cancer in which secondary tumors arise in distant organs such as lung, bone, and brain [2, 3]. During metastasis, cancer cells reorganize the cytoskeleton and reshaped themselves to bring the necessary changes in order to promote cell invasion [4, 5]. Invadopodia are the structures that appear transiently and responsible for extracellular matrix (ECM) degradation and cell invasion [6]; these are filamentous actin (F-actin)-based membrane protrusions that are rich in transmembrane type 1 matrix metalloproteinases (MT1-MMP) that aids in the degradation of ECM [7–9]. The formation of invadopodia is induced by various receptor tyrosine kinase and integrin signaling pathways. The signaling pathways converge on Src kinase, Rho GTPase and F-actin nucleation promoting factors [10, 11]. These events require the cytoskeletal regulators Cdc42 and Rho, their effectors such as neural Wiskott–Aldrich syndrome protein (N-WASP)/WASP-family verprolin-homologous protein (WAVE) [4, 12], and direct inducers of actin polymerization such as actin-related protein-2/3 (Arp2/3) [13]. Fer/CIP4 homology-Bin/Amphiphysin/RVS (F-BAR) belongs to bar domain protein subfamily [14]. Various members of the family have been recognized for their role in membrane remodeling events that occur in a variety of cellular pathways, such as cytokinesis, endocytosis, cell invasion. [15].

Previously, we have reported the role of Toca-1 in breast cancer invasion and metastasis [18]. Here, we found that expression of FBP17 was increased in breast cancer cell lines. Endogenous FBP17 localized to the leading edge of migrating cells and to invadopodia in cells invading gelatin. Since FBP17 serves as a scaffolding protein for Cdc42, Src, and N-WASp, we further tested the interaction of FBP17 with other actin regulatory proteins. We tested whether loss of FBP17 could result in decreased function of actin regulatory proteins (dynamin, cortactin, Arp2/3) that accumulate and promote invadopodia formation in MDA-MB-231 cells. Stable silencing of FBP17 in these cells led to a significant reduction in ECM degradation.

✉ Harish Chander
harish.chander@cup.edu.in

[1] Laboratory of Molecular Medicine, Department of Human Genetics and Molecular Medicine, Central University of Punjab, Bathinda 151001, India

Materials and methods

Cell lines and cell culture

Cell lines used in this study were acquired from National Centre for Cell Science (NCCS), Pune, and some were gifted by Andrew Craig, Queen's University, Kingston, Canada. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was used for MCF-7, SKBR3 and MDA-MB-231 cells. RPMI 1640 supplemented with 10% FBS was used for HBL-100, T47D, and ZR751 cells. MCF10A cells were grown in DMEM Nutrient Mixture F-12 HAM (10% FBS, 0.01 mg/mL transferrin, 0.02 mg/mL insulin, 25 nM sodium selenite, 50 nM hydrocortisone, 1 ng/mL EGF, 0.01 mM ethanolamine, 0.01 mM phosphorylethanolamine, 0.5% (w/v) bovine serum albumin (BSA), 0.5 mM sodium pyruvate, and 50 pM triiodothyronine).

Immunoblotting

Anti-FBP17 (NBP1-47260) was purchased from Novus Biologicals, anti-GAPDH (ZG003) was purchased from Invitrogen, and goat anti-mouse IgG (Cat# 115-035-003) and goat anti-rabbit IgG (Cat# 111-035-003) were purchased from Jackson Immuno research. Cells were harvested and lysed with NP-40 Lysis buffer (NaCl, 150 mM, NP-40, 1.0%, Tris–Cl 50 mM, pH 8.0) with protease inhibitor cocktail (Sigma) and phenylmethylsulfonyl fluoride (PMSF) (Sigma). Protein was estimated through Bradford Reagent (Bio-Rad) and Bovine Serum Albumin (BSA) as a standard (Bio-Rad). Cell lysates were run through SDS–PAGE, and buffers were purchased from Bio-Rad. Proteins were transferred using Amersham Protran Premium 0.2 µM Nitrocellulose Blotting membrane (Germany) through Trans Turbo Blotter (Bio-Rad). The membrane was incubated with primary and secondary antibodies, respectively, and immunoblot was developed in Chemi-Doc (Bio-Rad) using ECL (Bio-Rad).

RNA isolation and quantitative real-time PCR

Total RNA extraction was done using the RNA Isolation Kit (Sigma) according to the manufacturer's instructions. cDNA synthesis was done with Superscript-2 reverse transcriptase (Invitrogen) and random hexamer primers. qRT-PCR was performed using qRT-PCR Kit (Sigma) on cDNA using a human FBP17 primer (Forward 5-CTCTGGGATCAGTTT GACAACTT-3 Reverse 5-TGCCCTGCGTAATCATTC ATT-3) and GAPDH (Forward 5-GCCTTCCGTGTCCCC ACTGC-3 Reverse 5-CAATGCCAGCCCCAGCGTCA-3) primers and SYBR Green JumpStart Taq Ready Mix kit (Sigma-Aldrich; 58 °C annealing, 40 cycles, Applied Biosystems Cycler). Transcript levels were analyzed using the $2^{-\Delta\Delta CT}$ method [16].

Stable silencing of FBP17

For silencing of FBP17 in MDA-MB-231 cells, a system for lentiviral delivery of FBP17 shRNA was previously described [11]. Briefly, MDA-MB-231 cells were transduced with lentiviruses containing shRNA (GCATGA AGTTATCTCCGAGAA) against FBP17 and stable clones were selected with puromycin. The lysates from the stable clones were subjected to immunoblotting for the detection of FBP17 using beta-actin antibody as a loading control.

Co-immunoprecipitation

To check whether FBP17 associates with actin regulatory proteins in MDA-MB-231 cells, we have performed co-immunoprecipitation studies. Antibodies used in this experiment are mouse anti-cortactin (Upstate Biotech, Lake Placid, NY, USA), mouse anti-dynamin (Upstate Biotech.), and anti-arp2/3 (Upstate Biotech.). To identify FBP17-associated proteins, MDA-MB-231 cells were grown to 70% confluency. Then, the cells were harvested and lysed, subjected to Co-IP with rabbit anti-FBP17 for the detection of its interacting partners. Following recovery of IP complexes using Protein A-Agarose beads (Roche, Germany) and washing, bound proteins were resolved by SDS–PAGE. The protein was transferred into Nitrocellulose Blotting membrane (Amersham, Germany) using Trans Turbo Blotter (Bio-Rad), and immunoblotting was performed with indicated antibodies and the HRP-conjugated secondary antibodies. The NCP membranes were developed by using ECL (Bio-Rad) and detected using Chemi-doc (Bio-Rad).

Extracellular matrix degradation assays

MDA-MB-231 and FBP17 depleted cells were plated on thin ECM and incubated for 24 h before staining them with Alexa-488 Phalloidin.

MDA-MB-231 control and FBP17 depleted cells were seeded on TRITC-FN-coated glass coverslips (15,000 cells) containing a layer of thin gelatin [4]. After incubation for 24 h, cells were fixed, permeabilized, and stained with Alexa Fluor-488 Phalloidin (Life Technologies Inc., Burlington, ON, Canada). Epifluorescence microscopy was performed, and the areas of ECM digestion beneath the cells that were stained with Phalloidin were quantified as digestion area per cell using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). At least 100 cells were quantified for each condition and cell line.

Results

Expression of FBP17 in breast cancer cells

To determine the expression of formin-binding protein 17 (FBP17) in breast cancer cell lines, the basal levels of mRNA and protein were examined in various breast cancer cells and compared with normal cells. In order to determine the status of FBP17 protein, the cell lysates were subjected to immunoblotting and the membrane was probed with the anti-FBP17 antibody. Western blotting revealed the higher FBP17 levels in cancer cell lines as compared to normal like cells. Among the cancer cell lines, FBP17 was found to be highest in the invasive cell line MDA-MB-231. Densitometric analysis further confirmed the higher levels of protein expression of FBP17 in breast cancer cells (Fig. 1a, b). The qRT-PCR analysis also showed the higher levels of FBP17 transcripts in breast cancer cell lines (ZR751, MCF7, T47D, SKBR3, and MDA-MB-231) as compared to normal cell lines (MCF10A and HBL-100) (Fig. 2).

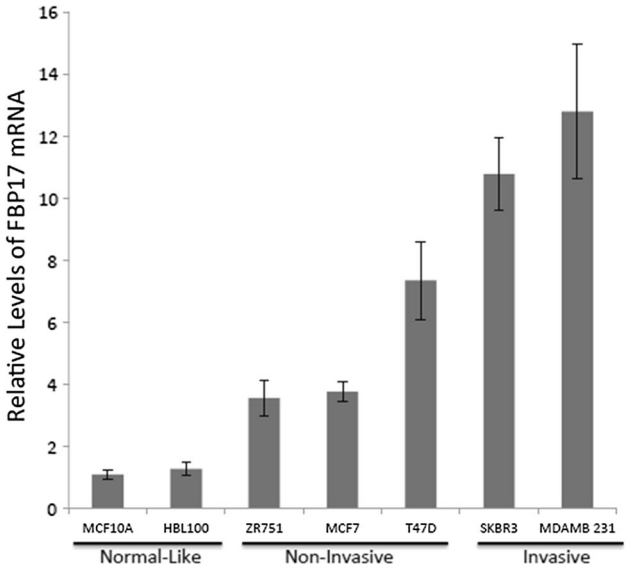
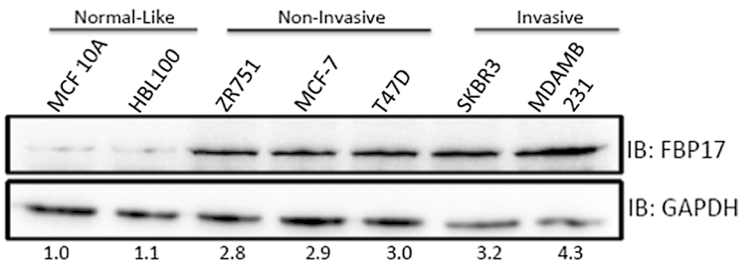


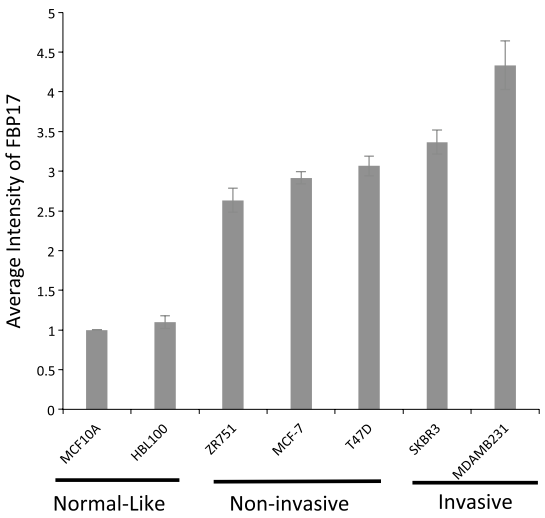
Fig. 2 Relative mRNA levels of FBP17 in breast cancer cells: quantitative RT-PCR analysis of FBP17 mRNA expression in MCF10A, HBL100, ZR751, MCF-7, T47D, SKBR3, and MDA-MB-231 cells. $2^{-\Delta\Delta}$CT values for FBP17 were normalized to GAPDH

FBP17 interacts with actin regulatory proteins

F-BAR proteins are involved in actin regulatory network including FBP17. To study the interaction of FBP17 with the proteins involved in actin regulation, we grew MDA-MB-231 cells and established stable FBP17 knockdown (KD) cell pools using short hairpin RNAs (shRNAs) as previously described [17]. We observed an almost complete depletion of FBP17 protein in lysates from MDA-MB-231-KD cells and compared with the levels in vector control MDA-MB-231-Non-Targeting (NT) cell lysates as revealed by immunoblotting using with the FBP17 antibody (Fig. 3a). We employed immunoprecipitation studies using the cell lines MDA-MB-231 and MDA-MB-231 KD (FBP17 knock down) by shRNA. Antibody against FBP17 immunoprecipitated FBP17 as shown in the immunoblotting experiment (Fig. 3b). The co-immunoprecipitates were subjected to immunoblotting with specific antibodies. These studies revealed the interaction of FBP17 with several binding



(a)



(b)

Fig. 1 Levels of FBP17 breast cancer cell lines: **a** Lysates from the breast cancer cell lines were subjected to immunoblot with FBP17 and GAPDH antibodies. **b** The graph depicts the higher expression of FBP17 as measured by densitometry in invasive breast cancer cells

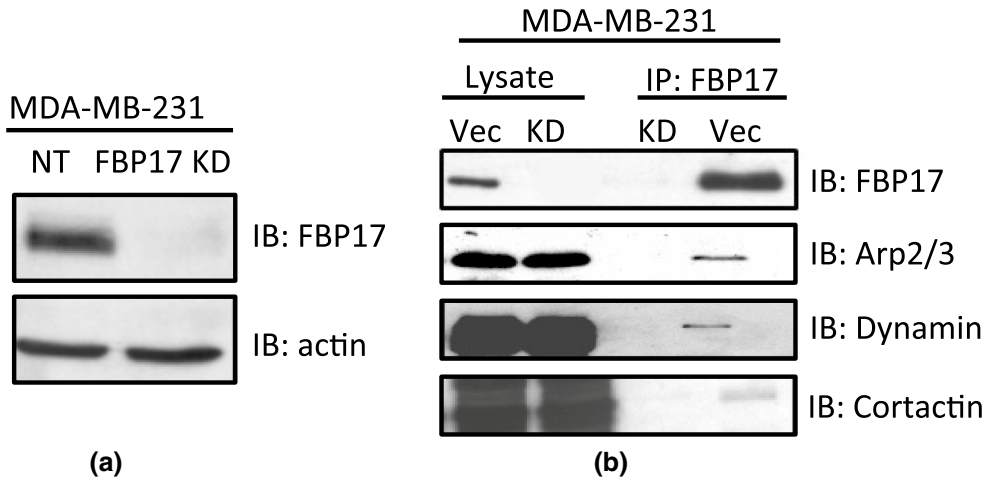


Fig. 3 Interaction of FBP17 with actin regulatory proteins in MDA-MB-231 cells: **a** MDA-MB-231 cells were transduced with lentiviruses containing shRNA against FBP17 and stable clones were selected with puromycin. Immunoblotting revealed the knockdown of FBP17. **b** Immunoprecipitation studies indicated that FBP17 interacts with actin regulatory proteins. MDA-MB-231 cells and FBP17 stable knockdown's lysates were subjected to immunoprecipitation with the FBP17 antibody. Immune complexes were subjected to immunoblotting with cortactin, dynamin, and Arp2/3 antibodies

partners including dynamin and cortactin. Additionally, p34 subunits of Arp2/3 were also detected in FBP17 Co-IPs. The controlled IP with MDA-MB-231 NT cells using the FBP17 antibody did not show any binding with these proteins (Fig. 3b). These results suggest the novel interaction of FBP17 with actin regulatory proteins and components of invadopodia such as cortactin.

FBP17 enhances ECM degradation

To determine the role of FBP17 in enhancing ECM degradation, MDA-MB-231 KD cells were plated on to ECM along with MDA-MB-231-NT cells as a control. After 24 h of incubation on ECM F-Actin (Alexa-488 Phalloidin) and DAPI, staining was performed. Epifluorescence micrographs of the cells showed the reduced degradation of ECM in FBP17 KD cells (Fig. 4a). Additionally, quantitative estimation of ECM degradation area per cell revealed a defect in the capability of FBP17 KD cells in degrading ECM compared to the parental cells (Fig. 4b). These results suggest that an important role for FBP17 in invadopodia formation and invasion of breast cancer cells.

Discussion

In this study, we report novel findings linking the F-BAR protein FBP17 in promoting invadopodias formation and invasion of breast cancer cells. Basal-like breast cancers are highly metastatic and induce invadopodia formation that induce degradation of basement membrane leading to metastasis. The present investigation identified FBP17 as a proinvasive regulator of breast cancer cells. Here, we report that the expression of FBP17 is higher in breast cancer cells as compared to normal cells. We also observed that FBP17 associates with actin regulatory proteins that are linked to invasion. During the invasion, the rearrangement of cytoskeleton takes place and actin regulatory proteins play an important role in shaping the cytoskeleton of invading cells [18]. Recently, many studies have suggested the role of F-BAR proteins in the invasion of cancer cells. F-BAR proteins direct the invasion by protein–protein interactions as recently reported for Toca-1 and CIP4 [19, 20, 21]. In our study, we report that FBP17, a scaffolding protein, is associated with actin regulatory proteins such as cortactin, dynamin and Arp2/3. Thus, the interaction of FBP17 with these proteins might be a regulatory factor in the formation of invadopodia. Recently, the role of FBP17 has been demonstrated in the pedestal formation by enterohemorrhagic *E. coli* [22]. Interestingly, we also found the role of FBP17 in invadopodia formation and enhancing the ECM degradation. Our findings have identified that FBP17 is overexpressed in breast cancer cells and suggests the role of FBP17 in the invasiveness of cancer cells. It is important to note that previous studies also suggested the upregulation of other F-BAR proteins in cancer cells. Gene expression profiling of invasive breast tumors showed high expression of Cdc-42 and Arp2/3 [19, 23]. There are high chances that upregulated proinvasive proteins require other adaptor proteins to enhance the ability of cancer cells to invade. It would be of interest to explore further what controls the expression of FBP17 in normal epithelial cells. The present study revealed the role of FBP17 as a key player in ECM degradation through its association with actin regulatory proteins.

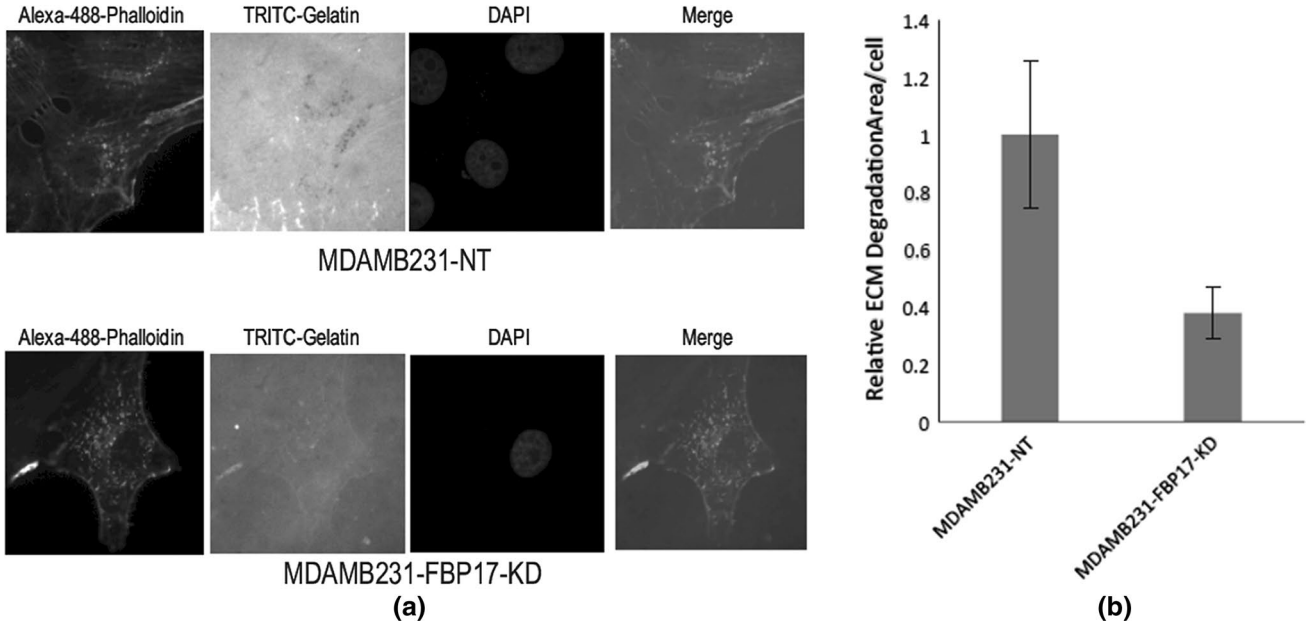


Fig. 4 FBP17 promotes ECM degradation: **a** MDA-MB-231 cells and FBP17 depleted cells were plated on thin ECM and incubated for 24 h before staining them with Alexa-488 Phalloidin. Upper panel shows the degradation of ECM by control cells. **b** Relative area of degradation per cell

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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