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MFAP5 promotes tumor progression and bone metastasis by regulating ERK/MMP signaling pathways in breast cancer

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ABSTRACT

Breast cancer accounts for about 30% of all cancers in women, while approximately 70% breast cancer patients developed bone metastases throughout the course of their disease, highlighting the importance of exploring new therapeutic targets. Microfibrillar-associated protein 5 (MFAP5) is a component of extracellular elastic microfibril which has been confirmed to function in tissue development and cancer progression. But the role of MFAP5 in breast cancer remains unclear. The present study demonstrated that MFAP5 was up-regulated in breast cancers compared with that in normal breast tissues, and further increased in breast cancer bone metastasis. Functionally, MFAP5 overexpression accelerated breast cancer cell proliferation and migration, while an opposite effect was observed when MFAP5 was knocked down. In addition, up-regulation of MFAP5 increased the expression of MMP2 and MMP9 and activated the ERK signaling pathway. Conversely, inhibition of MFAP5 suppressed the expression of MMP2, MMP9, *p*-FAK, *p*-Erk1/2 and *p*-cJun. These findings may provide a better understanding about the mechanism of breast cancer and suggest that MFAP5 may be a potential prognostic biomarker and therapeutic target for breast cancer, especially for bone metastasis of breast cancer.

1. Introduction

Breast cancer is the most commonly diagnosed malignant tumor in women, accounting for about 30% of all cancers in them [1]. Although technical advances in the treatment of cancer (including surgery, radiotherapy, chemotherapy and endocrine therapy) have reduced premature mortality of cancer patients remarkably [2], breast cancer still ranks second among the most common causes of cancer-related death in women [1]. Tumorigenesis is caused by various carcinogenic factors and characterized by uncontrolled cell proliferation and a high rate of metastasis [3,4]. Bone is the most common site of distant metastasis in patients with advanced breast cancer with an incidence of 60–80% [5–7], which is also the main cause for pathologic fracture and spinal cord compression, which seriously affect the quality of life of the patients [8,9]. But there are limited strategies to target bone metastasis of breast cancer, and therefore it is urgent to explore the molecular mechanism underlying breast cancer bone metastasis for developing new effective therapeutic targets.

Microfibrillar-associated protein 5 (MFAP5), also known as microfibril-associated glycoprotein 2 (MAGP2), is a component of extracellular elastic microfibril that functions in bone growth, cardiovascular development, alveolar elastogenesis and Marfan syndrome [10–12]. Studies [11,13] showed that MFAP5 was secreted by bone marrow mesenchymal stromal cells (BMSC), and functioned in hematopoiesis and immune systems, and that loss of MFAP5 is protective against bone loss in mice [11,13]. These findings suggest that MFAP5 works as a crucial factor in the bone microenvironment. Recent studies [14–16] further showed that MFAP5 was overexpressed in head & neck, pancreatic, lung and tongue cancers. But the role of MFAP5 in these cancers remains to be elucidated. Serum MFAP5 level was reported to be negatively correlated with

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prognosis of ovarian cancer patients, and MFAP5 secreted by cancer cells was found to promote tumor proliferation, endothelial cell motility, chemoresistance and angiogenesis [17–19]. However, there are few studies reporting the role of MFAP5 in breast cancer or bone metastasis.

Given the important role of MFAP5 in both tumor and bone microenvironments, we postulated that MFAP5 may play an important role in breast cancer progression and bone metastasis. The aim of the present study was to detect the expression MFAP5 expression in breast cancer bone metastasis, determine the effect of MFAP5 on cell proliferation and migration of breast cancer cells, and finally explore the possible underlying molecular mechanism, especially about the role of the ERK/MMP pathway in this process.

2. Materials and methods

2.1. Patients and specimens

Para-tumor normal breast tissues (n = 40), primary breast cancer specimens (n = 54) and bone metastasis tissues (n = 40) were collected from breast cancer patients who received surgical resection in Changzheng Hospital of the Second Military Medical University (Shanghai, China). Among the clinical samples, matched specimens from three patients (normal breast tissue, primary cancer specimen and bone metastasis tissue were from the same patient) were collected. Written informed consent was obtained before sample collection. The study protocol was approved by the Institutional Review Board of the said university. The tissue specimens were divided into two: one was snap-frozen and stored in liquid nitrogen, and the other was fixed in formalin and paraffin embedded. All the sample treatment procedures were completed within 2 h after surgical excision.

2.2. Immunohistochemistry

The sample was fixed with 4% paraformaldehyde, dehydrated through a graded series of ethanol, paraffin embedded, and sliced into 5-μm sections. Immunohistochemical (IHC) staining for MFAP5 (ab171737, Abcam, USA) was carried out using standard histological procedures described in the manual for Histostain-Plus (DAB) kit (Mingrui Biotech, China). Staining was recorded in accordance by two investigators with previous protocols as high or low blindly.

2.3. Western blot

Total protein (100 μg) was separated using SDS-PAGE and then transferred onto nitrocellulose membranes (Thermo, USA). The blotted membranes were incubated with antibodies for MFAP5 (ab171737), MMP2 (AF5330, Affinity, USA), MMP9 (AF5228, Affinity), *p*-FAK (Try861) (AF3399, Affinity), FAK (AF6397, Affinity), *p*-Erk1/2 (AF1015, Affinity), Erk1/2 (AF6240, Affinity), *p*-cJun (Ser63) (AF3089, Affinity), *p*-cJun (Ser73) (AF3095, Affinity), cJun (AF6090, Affinity), and actin (AF7018, Affinity) diluted at 1:1000. After washing, the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse antibodies (Santa Cruz, USA). The blots were developed using EasySee Western Blot Kit (TransGen Biotech, China). Beta-actin protein was also determined by using the specific antibody (Santa Cruz) as a loading control. All experiments were carried out in triplicate.

2.4. qRT-PCR assay

Total RNA was isolated by using TRIZOL (Invitrogen, USA) and reverse transcribed into cDNA by using Prime Script™ RT Master Mix (Takara, Japan). Gene transcripts were quantified on 7900HT Fast Real-Time PCR System (Life Technologies Corporation, USA) using SYBR Green qPCR Master Mix (Bimake, USA) and normalized with GAPDH. All primers are listed in Supplementary Table 1.

2.5. MFAP5 overexpression plasmid and siRNAs

For MFAP5 overexpression plasmid, vector pcDNA3.1+ plasmid was enzyme digested by *Kpn*I and *Eco*RV (TransGen Biotech, china) and the code sequence of MFAP5 amplified by PCR was inserted into pcDNA3.1+ by using Quick-Fusion Cloning Kit (Bimake). The PCR primer sequences were: S: AACTTAAGCTTGGTACATGTCGCTCTTGGGACC; A: GCCACTGTGCTGGATTCACAGACCATTGGGTCTC. Two different siRNA oligos against MFAP5 were purchased from Shanghai Genechem Co., Ltd (China). The sequences were: siRNA-1: CTTCACCAGTTTACGACGTAT; siRNA-2: GAAGATCCTAATCTGGTGAAT.

2.6. Cell culture and transfection

Two breast cancer cell lines MCF7 and MDA-MB-231 (Cell Bank of Type Culture Collection Committee of the CAS, Shanghai, China) were routinely maintained in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in a humidified air atmosphere containing 5% CO_2. Cells of logarithmic growth were transfected with plasmids using DNA Transfection Reagent (Bimake) or with siRNA using Lipofectamine™-2000 (Invitrogen) when they grew to 50–70% confluence according to the manufacturer's instructions.

2.7. CCK8 assay

MCF7 and MDA-MB-231 cells transfected with overexpression plasmid or siRNA were seeded in 96-well plates at an initial density of 5×10^3 per well, cultured for 48 h, and assessed using the Cell Counting Kit 8 (Bimake). The results were measured by absorbance at 450 nm using an ELx800 microplate reader (BioTek Instruments Inc., USA).

2.8. Transwell assay

An 8-μm pore size transwell chamber (Corning, USA) was used for transwell migration assay. MCF7 and MDA-MB-231 cells transfected with overexpression plasmid or siRNA were digested and counted. A total of 1×10^5 cells in 100 μL medium supplemented with no FBS were plated in the upper chamber and 500 μL medium supplemented with 10% FBS was covered on the bottom chambers as chemoattractant. After 24-h incubation in a humidified incubator, non-migratory cells on the upper membrane surface were carefully removed, and those on the bottom surface were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet for 15 min. Cells were counted by photographing 5 random fields under a microscope at 400 × magnification.

2.9. Statistic analysis

SPSS 19.0 statistical software (SPSS Inc., Chicago, IL) was used for statistical analysis. Data are presented as mean ± standard error of the mean (SEM). Statistics of the mean value between groups were assessed using independent Student *t*-test, assuming double-sided variance. All experiments were repeated at least three times, and representative experiments are shown. *P* values of <0.05 were considered statistically significant.