Zinc finger Protein 143 expression is closely related to tumor malignancy via regulating cell motility in breast cancer

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SUPPLEMENTARY MATERIALS AND METHODS

Materials

Tissue array slides (CBA4) containing human breast cancer, metastatic, and normal tissues were purchased from SuperBioChips Laboratories (Seoul, Republic of Korea). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT, USA). Lipofectamine® 2000 was obtained from Invitrogen™ (ThermoFisher Scientific, Waltham, MA, USA). Mouse monoclonal antibodies against β-actin, vimentin, and ZNF143 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against MMP13 was obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies specific for E-cadherin, and horseradish peroxidase-conjugated anti-mouse and anti-

rabbit antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody against ZEB1 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Short-hairpin (sh) RNA-lentiviral particles against human ZNF143 and its control were obtained from Santa Cruz Biotechnology. . Rhodamine-phalloidin was obtained from Molecular Probe ™ (Thermofisher Scientific. Ltd.)

Immunohistochemistry and HistoQuest® Analyses

ZNF143 staining on tissue arrays (SuperBioChips Laboratories, slide CBA4) was conducted by the National Cancer Center (NCC) Animal Sciences Branch. Breast tissues in the tissue microarrays were stained with hematoxylin and mouse anti-ZNF143 antibody (1:200, Santa Cruz Biotechnology) and detected with 3,3′-diaminobenzidine (DAB). The stained tissues on the tissue microarray were digitized at 20 × magnification using an Aperio AT Turbo whole slide scanner (Leica Biosystems, Buffalo Grove, IL, USA) equipped with a clinical grade RGB camera. Manually delineated regions of interest (ROI) were extracted from the digitized slide. A total of 90 tumor and normal images from 42 tumors and 7 normal tissues were collected and exported as 3,200 × 2,800 pixel matrices in TIFF format. These images were used to extract features such as the intensities of ZNF143 and hematoxylin by using HistoQuest® analysis software (TissueGnostics, Vienna, Austria), which quantitated the percentage of ZNF143-positive nuclei (weak, moderate, and strong intensities) relative to the total number of hematoxylin-positive nuclei per tumor. Images from the slide which was stained without primary antibody were for negative control.

Cell culture

Human breast cancer MCF7 cells (HTB-22) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained as monolayers in RPMI 1640

medium supplemented with 10 % heat-inactivated FBS. All cells were grown at 37 °C in a humidified 5 % CO_2 atmosphere.

The short-hairpin (sh)RNA-mediated silencing of human ZNF143 in breast cancer cells

To achieve stable lentivirus-mediated expression of shRNA specific for the gene encoding ZNF143 in MCF7 cells, cells were grown for 24 h, incubated with 5 μg/mL polybrene for 30 minutes, and then infected as previously described (21). To avoid clonal variations, the individual clones for each stable cell line produced by infection were pooled. Established cell lines were propagated and their images were captured using an inverted light microscope (CKX53; Olympus, Tokyo, Japan).

Transfection of human ZNF143

MCF7 sh-ZNF143 cells were transfected with pFLAG-CMV2-hZNF143FL or an empty vector using Lipofectamine® 2000, grown for 24 h, and harvested for the migration, invasion, and immunoblotting assays.

Scanning electron microscopy (SEM) of the cell morphology

Cells were fixed in 0.1 ml/L sodium phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C. The cells were then washed and dehydrated using graded alcohol (50 % – 100 %). Then, the cells were serially treated with 25 %, 50 %, and 75 % isoamyl acetate in ethanol, and finally with 100 % isoamyl acetate. The cells were then dried by critical point drying (HDP-2; Hitachi, Tokyo, Japan) at Eulji University, coated with platinum, and observed using SEM (Hitachi S4700) at Eulji University.

F-actin staining

Subconfluent breast cancer cells were fixed with 4 % paraformaldehyde for 15 minutes and rinsed three times with phosphate-buffered saline (PBS). The cells were then stained with rhodamine-phalloidin for 30 minutes, followed by 4′,6-diamidino-2-phenylindole (DAPI) treatment for 10 minutes in the dark at room temperature. After washing with PBS, digital images of the cells were obtained at 20 × magnification using a Cytation 3 (BioTek Instruments, Winooski, VT, USA). The images were obtained at 60 × magnification using a DeltaVision OMX™ SR (GE Healthcare, Sunnyvale, CA, USA).

Wound healing assay

Cells (5×10^5) were grown in 12-well plates for 24 h until they were fully confluent. A uniform scratch was then made and the plate was washed with PBS. Digital images of the wound were obtained every 12 h at 10 × magnification using the NCC cell observer system (Axiovert 200M; Carl Zeiss, Oberkochen, Germany). The horizontal distance between the two sides of the wound was then measured (21).

Transmigration and invasion assays

Cells were suspended in RPMI 1640 medium and placed in the upper compartment of an 8 μm Transwell® (3.2 mm diameter; Neuro Probe, Gaithersburg, MD, USA). The lower compartment was filled with RPMI 1640 medium supplemented with FBS. After 24 h, the filter was washed with PBS and the migrated cells on the filter membrane were stained using a Diff-Quik Stain Kit (Sysmex, Tokyo, Japan). For invasion assays, the upper compartment of an 8 μm Transwell® (6.5 mm diameter; Costar, Cambridge, MA, USA) was coated with Matrigel® (1 mg/mL) before starting the assay. Each assay was conducted at least three times, and three random fields using 20 × magnification were analyzed for each filter membrane (21).

Isolation of RNA and RT-PCR

Total cellular RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA) and dissolved in diethyl pyrocarbonate-treated water (37). The RNA was quantified by UV scanning, and samples (5 μg) were reverse-transcribed in the presence of oligo(dT) primers as described previously (23). The identities of the primers for each gene are available upon request (21, 29). The PCR products were subjected to electrophoresis and the resulting bands were visualized with ethidium bromide and photographed using Gel Doc software (Bio-Rad, Hercules, CA, USA).

Immunoblotting

Protein samples were resolved by SDS-PAGE, followed by protein transfer to polyvinylidene-difluoride membranes for 1 h using a Bio-Rad transfer unit (Bio-Rad). The membranes were then blocked for 30 minutes in Tris-buffered saline containing 0.01 % Tween 20 (TBST) and 5 % nonfat dried milk, followed by incubation for 2 h with primary antibody in TBST containing 2 % bovine serum albumin, and then 1 h of incubation with horseradish peroxidase-conjugated anti-mouse or rabbit antibody. The blots were developed with WEST-ZOL® plus western blot detection system (Intron Biotechnology, Daejeon, Republic of Korea). Quantitation of band intensities on the XAR-5 film (Eastman Kodak, NY, USA) was performed using Quantity One software (Bio-Rad)(21, 38).

TCGA provisional analyses

For the analyses of matrix metallopeptidase 13 (MMP13) from breast cancer patients, we first gathered gene expression data from RNA-Seq data at the Cancer Genome Atlas (TCGA) data portal. The gene expression data were normalized by RNA-Seq expression estimation using expectation-maximization (RSEM) (39). To determine the differential expression

between normal and tumor tissues, boxplots were drawn and the significance was determined using the Student's *t*-test and R software (https://www.r-project.org/about.html). To perform the survival analyses, clinical information of breast cancer patients was recorded and annotated according to gene expression levels by our own scripts, and then analyzed by the Kaplan-Meier method using the R software.

Statistical analyses

All data are expressed as percentages of the control and are shown as means ± S. E.. Student's *t* test was used to make statistical comparisons between groups. Values of $p < 0.05$ were considered significant.