MAP3K1 expression is associated with progression and poor prognosis of hormone receptor-positive, HER2-negative early-stage breast cancer

Sung-Hsin Kuo, Ming-Feng Wei, Yi-Hsuan Lee,Wen-Chi Yang, Shi-Yi Yang,Jui-Chueh Lin, and Chiun-Sheng Huang

**Supplementary Methods**

**Cell proliferation and viability assay**

For cell proliferation assay, an equal number of cells of both breast cancer cell lines, stably expressing either shMAP3K1 (MAP3K1 shRNA transfection) or scrambled shRNA, were plated in a 96-well plate. At first, third, fifth, and seventh days after cell seeding, cell growth was estimated using the tetrazolium bromide (MTT) assay. The absorbance of each sample was compared with first day values. The relative ratio of absorbance on Day 1 was arbitrarily presented as 100% for each treatment.

For cell viability assay, cells were plated at a concentration of 3,000 cells/well in 96-well plates. The next day, cells were incubated with either tamoxifen, doxorubicin, or docetaxel at various doses. At 72 h after drug treatment, cell viability was measured using MTT assay. Cytotoxicity was expressed as absorbance percentage of the control group without treatment. The data were expressed as the mean ± standard deviation (SD) of three different experiments. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

**Migration and invasion assay**

Cell migration was assessed by wound-healing assay using Culture Inserts (Ibidi, Martinsried, Germany). The breast cancer cells with different treatments were plated on separate sides of the culture inserts for 24 h at the concentration of 5 × 104 cells/side.25 Then, the total migrating distance of cells from the edges was photographed and measured at five random points for each replicate using Adobe Photoshop CS4 software at 24 h after removing the insert. Invasion assays were conducted using BD Falcon Cell Culture Inserts (Transparent PET membrane with 8.0 µm pore size; Falcon REF 353097).25 Then, 2 × 105 cells were resuspended in serum-free media and seeded in matrigel-coated inserts. The outer chamber was filled with complete medium supplemented with 10% FBS. After incubation for 24 h at 37 °C, non-invading cells on the upper surface of the insert were removed using a cotton-tipped swab. The invading cells were fixed with cold methanol, stained with 0.5% crystal violet, and counted under a microscope.

**Cell cycle analysis**

Each cell of breast cancer cell lines attached to a 6-cm culture dish was harvested, washed twice with phosphate-buffered saline (PBS), and fixed in 70% ice-cold ethanol overnight. Fixed cells were washed, resuspended in cold PBS with 20 μg/mL RNAase, and incubated at 37 °C for 30 min. The cells were then stained with propidium iodide (PI; 50 μg/mL) and incubated on ice for 30 min. The distribution of cell cycle phases was determined using a fluorescence-activated cell sorting (FACS) flow cytometer (Becton-Dickinson), and the relative proportion of cells with DNA content indicative of G0/G1-phase, S-phase, and G2/M-phase was assessed using Cell Quest.26

**Apoptosis analysis using flow cytometry**

The number of apoptotic cells was determined using a FITC Annexin V Dead Cell Apoptosis Kit (V13242, Invitrogen, Waltham, MA, USA). Briefly, the untreated control cells, scramble control cells (pGIPZ vector), and shMAP3K1-transfected MCF7 and T-47D cells were washed with PBS, and then resuspended in 500 μL annexin-binding buffer at a concentration of 1 × 106 cells/mL. Subsequently, the cells were incubated with 5 μL Annexin V-FITC and 1 µL PI (100 μg/mL) at 37 °C in the dark for 15 min. The percentage of apoptotic cells was evaluated immediately using the FACSCalibur flow cytometer (Becton-Dickinson) [26].

**Luciferase assay**

For the NF-κB promoter analysis, MCF7 and T-47D cells were transfected with 5 µg of NF-κB-Luc reporter plasmid DNA, containing luciferase gene under the control of NF-kB promoter (BD Bioscience, Clontech, Palo Alto, CA, USA) for 6 h by using TransFast Transfection Reagent (Promega, Madison, WI, USA). The luciferase activity of the cell lysates was measured according to the luciferase assay kit manual (Promega) and normalized to the amounts of protein in each cell lysate [27].

**Immunoblotting analysis**

Whole-cell lysates and nuclear lysates were harvested from each cell subclone. Equal amounts of protein extracts were fractionated on sodium dodecyl sulfate (SDS)-Tris glycine polyacrylamide gel electrophoresis (PAGE) gel and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). Primary antibodies against the following molecules were used for the analysis: MAP3K1 (clone 256, Abgent, San Diego, CA, USA) [28], Bcl-2 (DAKO, Glostrup, Denmark; Code No. M887, Lot 063), Bcl-xL (#2764; Cell Signaling Technology, Danvers, MA), c-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin B1 (sc-752; Santa Cruz Biotechnology), cyclin D1 (#2972; Cell Signaling Technology), cleaved poly (ADP-ribose) polymerase (PARP; #5625; Cell Signaling Technology), p-IκBα (sc-8404; Santa Cruz Biotechnology), p52 (sc-848; Santa Cruz Biotechnology), ERK (sc-1674; Santa Cruz Biotechnology), p-ERK (phospho-p44/42 MAPK [Erk1/2] [Thr202/Tyr204] [20G11]; 4376; Cell Signaling, Danvers, MA), matrix metalloproteinase (MMP)-9 (ab74277, Abcam, Cambridge, UK), BCL3 (sc-185; Santa Cruz Biotechnology), p65 (sc-7151; Santa Cruz Biotechnology), α-tubulin (CP06, Calbiochem, San Diego, CA, USA), β-actin (A5316, Sigma-Aldrich, MO, USA), and Ku80 (#2180; Cell Signaling Technology) [27, 29, 30]. The specific reactive bands on membranes were probed using appropriate secondary IgG antibodies conjugated to horseradish peroxidase. The immune complexes were visualized using an enhanced chemiluminescence detection system (ECL, Boehringer Mannheim, Mannheim), and quantification was performed using the Image Quant software (GE Healthcare).

**References: in the main manuscript**