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

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Abstract
Purpose In this study, we assessed whether the overexpression of MAP3K1 promotes the proliferation, migration, and invasion of breast cancer cells, which affect the prognosis of hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative early stage breast cancer.
Methods Two HR-positive, HER2-negative breast cancer cell lines (MCF7 and T-47D) overexpressing MAP3K1 were transfected with two MAP3K1 short hairpin RNA plasmids (shMAP3K1 [#3] and shMAP3K1 [#5]). The proliferation, migration, and invasion of these cells were then examined. We assessed whether shMAP3K1 affects the cell cycle, levels of downstream signaling molecules (ERK, JNK, p38 MAPK, and NF-κB), and sensitivity to chemotherapeutic and hormonal agents. To assess the anti-tumor effect of MAP3K1 knockdown in the breast cancer orthotopic model, MCF7 and T-47D cells treated with or without shMAP3K1 (#3) and shMAP3K1 (#5) were inoculated into the mammary fat pads of mice. In total, 182 patients with HR-positive, HER2-negative T1 and T2 breast cancer and 0–3 nodal metastases were included. Additionally, 73 patients with T1 and T2 breast cancer and negative nodes who received adjuvant endocrine therapy alone were selected as an independent validation cohort.
Results In both cell lines, shMAP3K1 (#3) and shMAP3K1 (#5) significantly reduced cell growth, migration, and invasion by downregulating MMP-9 and by blocking the G2/M phase of the cell cycle and its regulatory molecule cyclin B1. Moreover, both shMAP3K1 (#3) and shMAP3K1 (#5) downregulated ERK-, JNK-, p38 MAPK-, and NF-κB-dependent gene transcription and enhanced the sensitivity of both cell lines to doxorubicin, docetaxel, and tamoxifen. We observed that both shMAP3K1 (#3) and shMAP3K1 (#5) inhibited tumor growth compared with that in the scrambled group of MCF7 and T-47D cell orthotopic tumors. Patients with MAP3K1 overexpression exhibited significantly poorer 10-year disease-free survival (DFS) (70.4% vs. 88.6%, p = 0.003) and overall survival (OS) (81.9% vs. 96.3%, p = 0.001) than those without MAP3K1 overexpression. Furthermore, phospho-ERK (p < 0.001) and phospho-JNK (p < 0.001) expressions were significantly associated with MAP3K1 expression, and both phospho-ERK and phospho-JNK expressions were significantly correlated with poor 10-year DFS and OS. These biological findings, including a significant association between DFS and OS, and the expressions of MAP3K1, phospho-ERK, and phospho-JNK were further validated in an independent cohort. Multivariate analysis identified MAP3K1 expression as an independent poor prognostic factor for DFS and OS.
Conclusion Our results indicate that the overexpression of MAP3K1 plays a major role in the poor prognosis of HR-positive, HER2-negative early stage breast cancer.

Keywords MAP3K1 · Breast Cancer · Hormone receptor-positive · NF-κB · Prognosis

1 Introduction
Breast cancer is the most common cancer in women worldwide [1]. In Taiwan, there has been a continuous rise in the incidence of breast cancer [2]. Based on gene expression
profiles, breast cancers are classified into different molecular subtypes [3, 4]. Using immunohistochemistry (IHC) to assess the expression patterns of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) and using fluorescence in situ hybridization (FISH) technique to assess the amplification of HER2 encoding gene in HER2 IHC (score: 2+) breast cancer, its subtypes can be categorized as follows according to the 2017 St. Gallen Consensus: HR-positive and HER2-negative; HR-positive and HER2-positive, HR-negative and HER2-positive, and triple-negative [5, 6]. Systemic adjuvant chemotherapy is routinely administered for providing the long-term benefits of decreasing recurrence and metastases and prolonging survival outcomes in patients with HER2-enriched or triple-negative subtypes of breast cancer [6–9]. For both HR2-positive and HER2-negative patients, systemic adjuvant chemotherapy and endocrine treatment are routinely administered for those with high-risk factors, such as high-grade, larger tumor size, and positive axillary lymph nodes (LN), whereas endocrine treatments are dispensed for those without high-risk factors [6–9].

In addition to clinicopathological features, several multi-gene assays, including MammaPrint®, Oncotype DX®, PAM-50 (Prosigna®), and EndoPredict® have been demonstrated to predict the survival outcomes of patients with HR-positive, HER2-negative, and LN-negative breast cancer to help physicians and patients in opting either adjuvant chemotherapy combined with endocrine therapy or endocrine therapy alone [10–13]. Considering that certain low-risk and high-risk patients still develop local recurrence and distant metastases despite receiving endocrine treatment or chemotherapy followed by endocrine therapy, respectively, the identification of novel genes that can act as alternative prognostic markers as well as targeted genes for HR-positive and HER2-negative patients is warranted.

Genome-wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs), such as MAP3K1 (Mitogen-Activated Protein Kinase Kinase Kinase 1) rs889312, associated with breast cancer risk [14–16]. We previously reported that MAP3K1 rs889312 is closely associated with poor disease-free survival (DFS) and overall survival (OS) in early-stage HR-positive breast cancer [17]. MAP3K1, also named as MEKK1 (MEK kinase 1), is a 196-kDa serine-threonine kinase and a member of the MAP3K family and the Ser/Thr protein kinase superfamily [18]. Growing evidence suggests that MAP3K1 participates in the MAPK signal transduction pathway, in response to several mitogenic and metabolic stimuli, including estrogen, which activates Jun amino-terminal kinases (JNK)1/2 (also known as MAPK8/9), extracellular-signal-regulated kinase (ERK)1/2, or nuclear factor kappa B (NF-κB) [19, 20]. The upregulation of the aforementioned molecules may promote cell survival and the development of HR-positive, HER2-negative breast cancer [20]. Besides, previous studies have demonstrated that MAPKs and MAPK phosphatase-1 (MKP-1) may be involved in resistance against drugs, including tamoxifen and other chemotherapeutic agents [21, 22].

Based on aforesaid evidences [19–22], we hypothesized that MAP3K1 can mediate the cell proliferation of HR-positive, HER2-negative breast cancer cells, and is possibly related to the resistance against the adjuvant tamoxifen and other chemotherapeutic agents, and thus, contributes to the early recurrence and metastasis of early-stage HR-positive, HER2-negative breast cancer. To prove this hypothesis, we investigated whether downregulation of MAP3K1 could inhibit cell proliferation, migration, and invasion, and affect ERK1/2, JNK, p38 MAPK, and NF-κB activity in the in vitro HR-positive, HER2-negative breast cancer cell lines. We further used MCF7 and T-47D breast cancer orthotopic models to evaluate whether knockdown of MAP3K1 can inhibit the tumor growth. To further elucidate the biological functions of MAP3K1, we assessed the relationship between MAP3K1 expression in tumor cells and clinical outcomes in patients with early-stage HR-positive, HER2-negative breast cancers. We also assessed whether the downstream effector molecule of MAP3K1, phospho (p)-ERK, and p-JNK correlated with the expression of MAP3K1 and the clinical outcome of the same group of tumors. The prognostic values of the aforementioned MAP3K1 and MAP3K1-related molecules, p-ERK and p-JNK, were further validated with an independent validation cohort.

2 Materials and methods

2.1 Cell lines, lentivirus production, and transduction

For the study, we used human HR-positive, HER2-negative breast cancer cell lines, MCF7 (ATCC® HTB-22™) and T-47D (ATCC® HTB-133™), to assess whether the inhibition of MAP3K1 can affect cell proliferation, cell migration, cell cycle, sensitivity to drugs, and NF-κB activity. These two breast cancer cell lines were cultured in either Eagle’s minimum essential medium (ATCC® 30-2003™ for MCF7) or RPMI-1640 medium (ATCC® 30-2001™ for T-47D) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), penicillin, and streptomycin (Flow Labs, Rockville, MD, USA). The cells were cultured in an incubator in a humidified atmosphere containing 5% CO2 at 37 °C.

The short hairpin RNA (shRNA)-expression vectors and MAP3K1 shRNA constructs were obtained from the National RNAi Core Facility (Taipei, Taiwan). For
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lentivirus packaging, pSPAX2, pMD2.G, and shRNA-expression vectors were co-transfected into 293T cells. The pGIPZ vector, containing a TurboGFP cassette, was used as a scrambled control and a tool for determining viral titer. The supernatant was harvested at 48 h after transfection, and then the lentiviral particles in supernatant were concentrated with PEG-it™ Virus Precipitation Solution (System Biosciences, CA, USA). We determined the titers of lentivirus by infecting 293T cells with varying concentrations of pGIPZ lentivirus. TurboGFP expression was assessed by flow cytometry, and the lentiviral titer was approximately 1 x 10^7 infectious units per mL (IFU/mL). The breast cancer cell lines, MCF7 and T-47D, were infected with virus at a multiplicity of infection (MOI) of 5. At 24 h after infection, these infected cells were screened with 2 µg/mL puromycin (InvivoGen, ant-pr-1).

Total RNAs were isolated from each cell subclone (control cells; scrambled cells; and cells transfected with five shMAP3K1 variants, namely, shMAP3K1 [#1], shMAP3K1 [#2], shMAP3K1 [#3], shMAP3K1 [#4], and shMAP3K1 [#5]) by using RNeasy Mini Kit (#74,106; Qiagen, Hilden, Germany). Reverse transcription reaction was performed using the Maxima first strand cDNA Synthesis Kit for real-time quantitative polymerase chain reaction (RT-qPCR; #K1641; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the expression of MAP3K1 was quantified using the KAPA SYBR FAST RT-qPCR kit (07959435001; Kapa Biosystems, Woburn, MA, USA) on Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems (Applied Biosystems; Thermo Fisher Scientific) and the gene expression was normalized against the β-actin mRNA level. The primers of MAP3K1 used for the RT-qPCR analysis were as follows: 5′-AGGTCGCACAGTGAAATCAG-3′ (forward), 5′-GTTTCCTCAGGGCATTATGGTG-3′ (reverse). The reactions were performed in triplicates.

### 2.2 Clonogenic survival assay, viability, migration, and invasion assay

The detailed information of clonogenic survival assay, viability, migration, and invasion assay are listed in the Supplementary Materials and methods [23].

### 2.3 Cell cycle analysis, apoptosis analysis, and luciferase assay

The detailed information of cell cycle analysis, apoptosis analysis, and luciferase assay are listed in the Supplementary Materials and methods [24, 25].

### 2.4 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), Bcl-2 (DAKO, Glostrup, Denmark; Code No. M887, Lot 063), Bel-xL (#2764; Cell Signaling Technology, Danvers, MA, USA), 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-1xBa (sc-8404; Santa Cruz Biotechnology), p52 (sc-848; Santa Cruz Biotechnology), ERK (sc-1674; Santa Cruz Biotechnology), p-ERK (phospho-p44/p42 MAPK [Erk1/2] [Thr202/Tyr204] [20G11]; #4376; Cell Signaling Technology), JNK (Stress-activated protein kinases (SAPK)/JNK; #9252; Cell Signaling Technology), p-JNK (SAPK/JNK [Thr183/Tyr185] [81E11]; #4668; Cell Signaling Technology), p38 MAPK (#9212; Cell Signaling Technology), matrix metalloproteinase (MMP)-9 (ab74277, Abcam, Cambridge, UK), BCL3 (sc-185; Santa Cruz Biotechnology), NF-κB (p65) (sc-7151; Santa Cruz Biotechnology), p-NF-κB (p65) (phospho S536, #ab86299, Abcam, Cambridge, MA, USA), α-tubulin (CP06, Calbiochem, San Diego, CA, USA), β-actin (A5316, SACO, USA), cyclin D1 (#2972; Santa Cruz Biotechnology), cyclin B1 (sc-752; Santa Cruz Biotechnology), cyclin A1 (#2764; Cell Signaling Technology), cleaved poly (ADP-ribose) polymerase (PARP; #5625; Cell Signaling Technology), and Ku80 (#2180; Cell Signaling Technology)

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).

### 2.5 Immunofluorescence analysis

Breast cancer cells with different treatments were plated on cell culture chamber slides (#30,104, SPL Life Sciences, Pocheon-si, Republic of Korea). The adherent cells were fixed for 8 min with 3.7% paraformaldehyde and subsequently blocked with 3% bovine serum albumin (9048-46-8, Sigma-Aldrich, Saint Louis, MO, USA) containing 0.3% Triton X-100 (9036-19-5, Sigma-Aldrich, Saint Louis, MO, USA) for 60 min at room temperature. After incubation with a primary antibody against p-NF-κB p65 (#3033; Cell Signaling Technology) overnight at 4 °C, the cells
were incubated with Alexa Fluor 488–conjugated secondary antibody (#A11034, Life Technologies, Carlsbad, CA) for 1 h at room temperature. Finally, the cell nuclei were stained with DAPI (#40,043, Biotium, Hayward, CA, USA) and mounted with fluorescent mounting medium (FMH030; ScyTek Laboratories, Logan, UT, USA). The stained cells were analyzed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

2.6 The animal experiment of orthotopic breast cancer model

Athymic female BALB/c nude mice (five to six weeks old) were purchased from the National Laboratory Animal Center, Taiwan. All experiment procedures on animal were revised and approved by the College of Medicine, College of Public Health, and Institutional Animal Care and Use Committee (No. 20170503) of National Taiwan University. The $1 \times 10^7$ MCF7 cells stably expressing scramble shRNA, shMAP3K1 (#3), and shMAP3K1 (#5) and the $1 \times 10^7$ T-47D cells stably expressing scramble shRNA, shMAP3K1 (#3), and shMAP3K1 (#5) were mixed with Matrigel® (354,248; Corning, NY, USA) and then orthotopically injected into the right mammary fat pad 7 days after the 17β-estradiol pellets (NE-131; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously on the left side of the neck. Body weight and tumor volume were measured once per week. We generated three groups of five mice for each group in the MCF7 cell orthotopic model and generated three groups of five mice for each group in the T-47D cell orthotopic model. Tumor volumes were calculated using the Eq. $0.5 \times L \times W^2$, where L is the length, and W is the width. At the end of the experiment, the mice were sacrificed, and individual tumor weights were determined.

2.7 Characteristics, treatments, and tissue samples of HR-positive, HER2-negative early breast cancer patients

Patients diagnosed with stage I or II (AJCC 2007) HR-positive, HER2-negative early breast cancers at the National Taiwan University Hospital between January 1, 1994, and June 30, 2006, were enrolled for the study. Patients were considered HR-positive if the percentage of ER- or PR-positive epithelial cells was $\geq 1\%$ [6, 17]. HER2 expression was measured using the universal iView-Dab detection kit. Scores of 0 and 1+ were considered negative, and a score of 3+ was considered positive. Gene amplification through FISH-based PathVysion assay (Vysis Inc., Des Plaines, IL, USA) was performed for tumors with a score of 2+. For the HER2 gene: chromosome 17 ratio of $\geq 2.0$, tumors were considered positive based on the American Society of Clinical Oncology guidelines [29]. Tumors were considered HER2-positive for an IHC score of 3+ or 2+, determined through FISH-based gene amplification.

Pathological and clinical information about treatment (including type of surgery, receipt or non-receipt of adjuvant systemic therapy, and type and dose of adjuvant systemic therapy) and follow-up information (including recurrence and distant metastasis) were obtained from pathology reports and clinical records. Patients with high-risk factors, such as grade III cancers, large tumors, lymphovascular invasion (LVI), and lymph node (LN) positivity (N1), received standard adjuvant chemotherapy, such as CMF (cyclophosphamide, methotrexate, fluorouracil), CEF (cyclophosphamide, epirubicin, fluorouracil), CAF (cyclophosphamide, adriamycin [doxorubicin], and fluorouracil), AC/EC, or AC/EC followed by paclitaxel/docetaxel regimens as defined in our previous study [8]. All enrolled patients received adjuvant endocrine therapy, with drugs such as tamoxifen [8]. Adjuvant radiotherapy was administered to all patients after breast conservation surgery [8]. After surgery and adjuvant therapy, the patients were regularly followed-up in our clinic. If patients were lost during follow-up, information on their disease status and survival was obtained from the patients’ charts, hospital cancer registry records, and the National Death Registry.

The immunohistochemical analyses of MAP3K1 protein expression and detailed demographic information were obtained from the patients and their medical charts with their written informed consent. The pathologic review and immunohistochemical studies were approved by the National Taiwan University Hospital (NTUH) ethics committee (Institutional Review Board [IRB] Number: 201804056RINB). The patients’ medical data were anonymized before access and analysis.

2.8 Immunohistochemistry analysis

The immunohistochemistry staining for MAP3K1 (MEKK1, #PA5-15085, Thermo Fisher Scientific Inc. MA, USA) [26], p-ERK [28], and p-JNK [30] were performed on paraffin-embedded sections of surgical specimens using an indirect immunoperoxidase method, according to manufacturer’s instructions. To confirm the specificity of MAP3K1, staining was performed on paraffin-embedded sections in the absence of the first, the second, or both the primary antibodies as negative controls. All sections were observed under a light microscope. The percentages of MAP3K1-positive cells (tumor cells with readily visible brown staining distinctly marking the tumor cell nucleus) were averaged to yield an immunohistological score ranging from 0 to 100%. The results were classified into two groups according to the intensity and extent of staining: in the MAP3K1-negative group in the MCF7 cell orthotopic model and generated three groups of five mice for each group in the T-47D cell orthotopic model and generated three groups of five mice for each group in the T-47D cell orthotopic model.
group, either no staining was present (staining intensity score: 0) or mild immunostaining or positive staining was detected in < 20% of the cells (staining intensity score: 1), and in the MAP3K1-positive group, moderate or strong immunostaining was present in 20–40% (staining intensity score: 2) or more than 40% of the cells (staining intensity score: 3). For the p-ERK marker and p-JNK, positive expression was defined as positive nuclear staining of p-ERK or p-JNK in ≥ 20% of tumor cells [30, 31].

2.9 Patients, treatment, and tissue samples for the independent validation cohort

Patients diagnosed with T1-T2 disease with negative axillary lymph nodes (AJCC 2007) and HR-positive, HER2-negative early-stage breast cancer who received adjuvant hormone therapy but no adjuvant chemotherapy at our institute between January 1, 2008, and June 30, 2012, who had events including locoregional recurrence, distant metastases, and death, and matched the clinicopathological features of patients without events, were selected as the independent validation cohort. This independent validation cohort comprised 73 patients, and the adjuvant endocrine therapy consisted of mostly tamoxifen and few patients receiving gonadotropin-releasing hormone agonists plus tamoxifen, or aromatase inhibitor (AI) for premenopausal women and tamoxifen or an AI for postmenopausal women. We further assessed the expression patterns of MAP3K1, p-ERK, and p-JNK in tumor specimens from this independent validation cohort to emphasize the biological significance of MAP3K1 in early-stage HR-positive and HER2-negative breast cancer.

2.10 Statistical analysis

In vitro experiments of the proliferation, migration, invasion, cell cycle, apoptosis assay, and luciferase assay, were repeated at least three times; the data of aforementioned assays were presented as the mean ± standard deviation (SD). The p values of the aforementioned experiments were determined using the student’s t test, and statistical significance was defined for a p value < 0.05. The association between MAP3K1, p-ERK, and p-JNK was analyzed by Spearman’s correlation.

For breast cancer patients, follow-up data available on December 31, 2014, were analyzed. DFS was measured from the date of the first surgery for breast cancer to local recurrence, distant recurrence, or death from any cause; OS was measured from the date of the first surgery to the date of death from any cause or the last follow-up date [32]. The DFS and OS were calculated using the Kaplan–Meier method, and the survival curves were compared using the log-rank test. In this study, all prognostic variables that were investigated in the univariate analysis, including tumor stage, tumor grade, axillary LN status, LVI, and MAP3K1, were also included in the multivariate analysis by using the Cox proportional hazards regression model. The clinical characteristics were compared using the chi-square test and Fisher’s exact tests. The p < 0.05 was considered statistically significant.

3 Results

3.1 Downregulation of MAP3K1 attenuates cellular proliferation, migration, and invasion of HR-positive, HER2-negative breast cancer cell lines

As shown in Supplementary Figs. S1 and S2, we used the target mRNA sequences and corresponding amino acid sequences of five shMAP3K1 variants (shMAP3K1 [#1], shMAP3K1 [#2], shMAP3K1 [#3], shMAP3K1 [#4], and shMAP3K1 [#5]) in this study to assess whether the efficiency of shMAP3K1 mRNA inhibition in both MCF7 and T-47D cells was prominent. Thereafter, we assessed the mRNA levels of MAP3K1 in control MCF7 and T-47D cells and in cells transfected with five shMAP3K1 variants by using RT-qPCR. We found that the mRNA expression levels of MAP3K1 were significantly downregulated in shMAP3K1 (#3) and shMAP3K1 (#5)–transfected MCF7 cells compared with MCF7 cells transfected with shMAP3K1 (#1), shMAP3K1 (#2), and shMAP3K1 (#4) (Supplementary Fig. S3). Similarly, the mRNA levels of shMAPK3-transfected T-47D cells and the mRNA expression of MAP3K1 were significantly downregulated in shMAP3K1 (#3) and shMAP3K1 (#5)–transfected T-47D cells (Supplementary Fig. S3).

To approve the specificity of MAP3K1, we used immunofluorescence, immunohistochemical analysis, and western blotting to detect MAP3K1 expression in control MCF7 and control T-47D cells (primary antibody alone, secondary antibody alone, and combination of primary and secondary antibodies), and in shMAP3K1 (#3)–transfected MCF7 cells and in shMAP3K1 (#3)–transfected T-47D cells (combination of primary and secondary antibodies). The results are illustrated in Supplementary Fig. S3.

Breast cancer cell lines expressing higher levels of MAP3K1 were transfected with shMAP3K1 to downregulate MAP3K1 protein expression (Fig. 1A). shMAP3K1 (#3) treatment significantly reduced the expression level of MAP3K1 by 90% in MCF7 cells (p < 0.001) and by 85% in T-47D cells (p < 0.001) compared with the scrambled group (Fig. 1A). In addition, shMAP3K1 (#5) treatment significantly reduced the expression level of MAP3K1 by 40% in
Clonogenic survival assay showed that the downregulation of MAP3K1 by shMAP3K1 (#3) decreased MCF7 cells by 77% ($p < 0.001$) and T-47D cells by 57% ($p < 0.01$) compared with the scrambled group (Fig. 1A).

Clonogenic survival assay showed that the downregulation of MAP3K1 by shMAP3K1 (#3) decreased MCF7 cells by 77% ($p < 0.001$) and T-47D cells by 57% ($p < 0.01$) compared with the scrambled group (Fig. 1A).
compared with scramble-transfected MCF7 and T-47D cells, respectively (Fig. 1B). In addition, shMAP3K1 (#5) decreased MCF7 cells by 62% (p < 0.01) and T-47D cells by 47% (p < 0.05) via clonogenic survival assay compared with scramble-transfected MCF7 and T-47D cells, respectively (Fig. 1B). The results of the migration assay showed that the number of migrated cells was reduced by 57.3% in shMAP3K1 (#5)–transfected MCF7 cells (p < 0.001) and by 90.3% in shMAP3K1 (#5)–transfected T-47D cells (p < 0.001) compared with scramble-transfected MCF7 and T-47D cells, respectively (Fig. 1C). Similarly, migrated cells was reduced by 31% in shMAP3K1 (#5)–transfected MCF7 cells (p < 0.05) and by 89.5% in shMAP3K1 (#5)–transfected T-47D cells (p < 0.01) compared with scramble-transfected MCF7 and T-47D cells, respectively (Fig. 1C). The results of the invasion assay showed that the number of invaded cells in shMAP3K (#3)–treated cells was reduced by 73.7% in MCF7 cells (p < 0.001) and by 63.1% in T-47D cells (p < 0.01) compared with scramble MCF7 and T-47D cells, respectively (Fig. 1D). Similarly, invaded cells were reduced by 57.3% in shMAP3K1 (#5)–transfected MCF7 cells (p < 0.01) and by 45.9% in shMAP3K1 (#5)–transfected T-47D cells (p < 0.01) compared with scramble-transfected MCF7 and T-47D cells, respectively (Fig. 1D). These findings indicated that the inhibition of MAP3K1 suppressed cell quantity, migration, and invasion in these two HR-positive, HER2-negative breast cancer cell lines.

3.2 Downregulation of MAP3K1 induces G2/M phase arrest and apoptosis and enhances drug sensitivity in HR-positive, HER2-negative breast cancer cell lines

Hu et al. reported that transfection with MAP3K1 small interfering RNA leads to the downregulation of expression of CDC25C and cyclin B1 (key molecules for G2/M transition during the cell cycle) in MCF7 cells and MCF-12 F cells (normal mammary epithelial cell line) [33]. In this study, we sought to assess whether shMAP3K1 could inhibit the cell number in both breast cancer cell lines by blocking programmed G2/M phase and downregulating cyclin B1.

After 48 h, we determined the distribution of the cell cycle phases in each cell line. As shown in Fig. 2A, in shMAP3K1 (#3)– and shMAP3K1 (#5)–transfected MCF7 cells, there was a significant increase in the number of cells in the G2/M phase of the cell cycle and, concomitantly, a significant decrease in the number of cells in the G0/G1 phase. By contrast, in T-47D cells, shMAP3K1 (#3) and shMAP3K1 (#5) treatments led to the arrest of a significant number of cells in the G2/M phase of the cell cycle. These findings indicate that shMAP3K1 blocked cell cycle progression in the G2/M phase. Annexin V staining revealed that shMAP3K1 (#3) treatment resulted in increased apoptotic events (early and late apoptosis level) in both MCF7 cells (13.79% ± 1.25% vs. 1.84% ± 0.42%, p = 0.002964) and T-47D cells (13.68% ± 0.31% vs. 4.87% ± 0.16%, p = 0.000517) compared with scrambled MCF7 and T-47D cells, respectively (Fig. 2B). Similarly, shMAP3K1 (#5) treatment caused increased apoptotic events (early and late apoptosis) in both MCF7 cells (5.78% ± 0.46% vs. 0.62% ± 0.06%, p = 0.001034) and T-47D cells (13.74% ± 2.1% vs. 5.05% ± 0.54%, p = 0.009155) compared with scrambled MCF7 and T-47D cells, respectively (Fig. 2B).

As shown in Fig. 2C, we found that the inhibition of MAP3K1 (#3) promoted a decrease in cell viability in tamoxifen-treated MCF7 (0.01, 0.1, 1, 10, and 50 μM) and T-47D (0.01, 0.1, 1, 10, and 100 μM) cells compared with cells transfected with scrambled shRNA. Furthermore, the inhibition of MAP3K1 (#3) increased cellular drug sensitivity to doxorubicin (0.01, 0.1, 1, 2, and 5 μM) in MCF7 cells, doxorubicin (0.01, 0.1, 1, 10, and 50 μM) in T-47D cells, and docetaxel (0.01, 0.1, 1, 10, and 20 μM) in both MCF7 and T-47D cells (Fig. 2D and E). Similarly, shMAP3K1 (#5) significantly decreased cell viability in tamoxifen-treated MCF7 (0.01, 0.1, 1, 10, and 50 μM) and T-47D (0.01, 0.1, 1, 10, and 100 μM) cells compared with cells transfected with scrambled shRNA (Fig. 2C). In addition, the inhibition of MAP3K1 (#5) increased the cellular drug sensitivity to doxorubicin (0.01, 0.1, 1, 2, and 5 μM) in MCF7 cells, doxorubicin (0.01, 0.1, 1, 10, and 50 μM) in T-47-D cells, and docetaxel (0.01, 0.1, 1, 10, and 20 μM) in both MCF7 and T-47-D cells compared with cells transfected with scrambled shRNA (Fig. 2D and E).

3.3 Downregulation of MAP3K1 significantly reduces the expression of cyclin B1 and anti-apoptosis-related factors, and NF-κB activity

We found that the expression of cyclin B1 was essentially reduced by shMAP3K1 (#3) transfection, while the expression of cyclin D1 (key molecule for G1 arrest during the cell cycle) was not affected (Fig. 3A). Considering that ERK, JNK, and p38 MAPK are downstream molecules of MAP3K1-signaling pathway [20], we assessed whether ERK, JNK, and p38 MAPK can be inhibited by MAP3K1 silencing. We found that shMAP3K1 (#3) transfection downregulated p-ERK, p-JNK, and p-p38 MAPK expression in both MCF7 and T-47D cells when compared with the scrambled group (Fig. 3A). In addition, we revealed that another shMAP3K1 (#5) transfection also downregulated the expression of p-ERK, p-JNK, and p-p38 MAPK expression in both MCF7 and T-47D cells (Supplementary Fig. S4).
S.-H. Kuo et al. transfection downregulated Bcl-2 expression in MCF7 cells, and Bcl-xL expression in both MCF7 and T-47D cells, whereas c-Myc expression remained unaltered (Fig. 3B).

Transfection with shMAP3K1 (#3) also led to increased expression of PARP and decreased MMP-9 expression (Fig. 3B). These results indicate that using shMAP3K1 to inhibit MAP3K1 can promote apoptosis and attenuate migration and invasion in these breast cancer cells.

NF-κB, which acts as a downstream factor in the MAP3K1 signaling pathway, is involved in the pathogenesis of HR-positive breast cancer cells [40]. As shown in Fig. 3C, transfection with shMAP3K1 (#3) inhibited the expression of p-IκBα, which is an essential regulator of NF-κB, and p-NF-κB (p65) in both MCF7 and T-47D breast cancer cells. Transfection with shMAP3K1 (#3) downregulated the nuclear expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in both MCF7 and T-47D cells. We found that shMAP3K1 (#3) transfection downregulated Bcl-2 expression in MCF7 cells, and Bcl-xL expression in both MCF7 and T-47D cells, whereas c-Myc expression remained unaltered (Fig. 3B). Transfection with shMAP3K1 (#3) also led to increased expression of PARP and decreased MMP-9 expression (Fig. 3B). These results indicate that using shMAP3K1 to inhibit MAP3K1 can promote apoptosis and attenuate migration and invasion in these breast cancer cells.

NF-κB, which acts as a downstream factor in the MAP3K1 signaling pathway, is involved in the pathogenesis of HR-positive breast cancer cells [40]. As shown in Fig. 3C, transfection with shMAP3K1 (#3) inhibited the expression of p-IκBα, which is an essential regulator of NF-κB, and p-NF-κB (p65) in both breast cancer cell lines. Transfection with shMAP3K1 (#3) downregulated the nuclear expression
MAP3K1 expression is associated with progression and poor prognosis of hormone receptor-positive, breast cancer cell lines (Supplementary Fig. S4). Further-more, fluorescence imaging showed that shMAP3K1 (#3) and shMAP3K1 (#5) significantly decreased the expression of p-NF-κB in both MCF7 and T-47D breast cancer cells. Cells were labeled with primary antibody against p-NF-κB and Alexa Fluor 488-conjugated secondary antibody. Cell nuclei were stained with DAPI. The image of p-NF-κB was observed using a fluorescent microscope. Scale bar: 10 μm (E). The results from three independent experiments using shMAP3K1 (#3)-transfected and shMAP3K1 (#5)-transfected MCF7 and T-47D cells are presented as RLU per milligram of protein (NF-κB-Luc promoter activity-luciferase assay) (**, p < 0.01). *p-NF-κB, the expression of p-NF-κB was measured from the total lysates of both MCF7 and T-47D breast cancer cell lines (Supplementary Fig. S4). Furthermore, fluorescence imaging showed that shMAP3K1 (#3) and shMAP3K1 (#5) transfection significantly decreased the expression of p-NF-κB in both MCF7 and T-47D breast cancer cells compared with breast cancer cells transfected with scrambled shRNA.
with scrambled shRNA (Fig. 3D). As indicated by the results from the NF-κB-Luc promoter activity assay, the transcription of NF-κB-dependent genes induced by the nuclear translocation and DNA-binding activity of NF-κB was decreased in both breast cancer cell lines after transfection with shMAP3K1 (#3) and shMAP3K1 (#5) (Fig. 3E).

Taken together, our findings indicated that MAP3K1 might play a role in promoting cell proliferation, anti-apoptotic function, migration, invasion, and NF-κB transcriptional activity, and thus, contributes to malignant progression and poor prognosis of HR-positive, HER2-negative breast cancer patients.

### 3.4 Inhibition of MAP3K1 expression reduces breast tumor growth in an orthotopic model

To assess the anti-tumor effect of MAP3K1 knockdown in the breast orthotopic model, MCF7 and T-47D cells treated with shMAP3K1 (shMAP3K1 [#3] and shMAP3K1 [#5]) or without shMAP3K1 transfection (scrambled shRNA) were inoculated into the right mammary fat pad of mice. We measured the tumor volume from the appearance of the initial tumor burden to the sacrifice phase each week. We found that there were no significant differences in body weight between the scrambled MCF7 group and the two shMAP3K1-transfected MCF7 groups (Fig. 4A). Similarly, we observed that there were no significant differences in body weight between the scrambled T-47D group and the two shMAP3K1-transfected T-47D groups.

We observed that both shMAP3K1 (#3) and shMAP3K1 (#5) inhibited tumor growth compared with the scrambled group of MCF7 cell orthotopic tumors (Fig. 4B). We showed that the shMAP3K1 (#3)–transfected group exhibited a 73.3% reduction in tumor volume (49.35 ± 11.09 mm³ vs. 184.81 ± 29.97 mm³, \( p = 0.0028 \)) and an 81% reduction in tumor weight (25.78 ± 3.94 mg vs. 135.76 ± 20.78 mg,

![Fig. 4](https://example.com/fig4.png)

Fig. 4 Silencing of MAP3K1 significantly inhibits tumor growth of MCF7 and T-47D breast cancer cell in an orthotopic model. (A). There were no differences in body weight among scrambled group, shMAP3K1 (#3)–transfected group, and shMAP3K1 (#5)–transfected group in both MCF7 cells– and T-47D cells– orthotopic models (B). Representative image showed that the orthotopic tumor sizes were suppressed in shMAP3K1 (#3)–transfected group and in shMAP3K1 (#5)–transfected group in both MCF7 cells– and T-47D cells– orthotopic models (C). In MCF7 cells–orthotopic models, both shMAP3K1 (#3)–transfected group (\( p < 0.01 \)) and shMAP3K1 (#5)–transfected group (\( p < 0.01 \)) caused significant reduction of tumor volume compared with scrambled group. In T-47D cells–orthotopic models, both shMAP3K1 (#3)–transfected group (\( p < 0.01 \)) and shMAP3K1 (#5)–transfected group (\( p < 0.01 \)) caused significant reduction of tumor volume compared with scrambled group. The results of tumor volume were expressed as \( n = 5 \) in each treatment group and measured at 5 weeks (D). Examples of tumor when mice were sacrificed 5 weeks after implantation. Scale bar: 10 mm (E). When compared with scrambled group at sacrificed phase, shMAP3K1 (#3)–transfected group and shMAP3K1 (#5)–transfected group all resulted in significant reduction of tumor weight in both MCF7 cells– and T-47D cells– orthotopic models (F). The expression patterns of MAP3K1, p-ERK, p-JNK, and p-NF-κB in tumor samples of MCF7 cells–orthotopic model (scrambled group vs. shMAP3K1 (#3) group vs. shMAP3K1 (#5)) (left panel). The expression patterns of MAP3K1, p-ERK, p-JNK, and p-NF-κB in tumor samples of T-47D cells–orthotopic model (scrambled group vs. shMAP3K1 (#3) group vs. shMAP3K1 (#5)) (right panel)
MAP3K1 expression is associated with progression and poor prognosis of hormone receptor-positive, HER2-negative breast cancer

3.5 Expression of MAP3K1, p-ERK, and p-JNK in tumor cells of patients with early-stage HR-positive, HER2-negative breast cancer

To further validate the biological significance of involvement of MAP3K1 in proliferation, local recurrence, and metastases of HR-positive, HER2-negative breast cancer, we assessed the relationship between expression of MAP3K1 in tumor cells, and the DFS and OS of 182 patients with either T1 or T2 status and negative or 1 to 3 lymph nodal metastases of HR-positive, HER2-negative breast cancer. As shown in Table 1, the median age was 49 years (range 23–81 years). The clinicopathological characteristics and treatments are listed in Table 1. All ER-positive and/or PR-positive patients received adjuvant endocrine therapy; nineteen patients (10.4%) received ovarian ablation or a luteinizing hormone-releasing hormone agonist with or without tamoxifen, and 163 (89.6%) patients received tamoxifen. None of the patients received an aromatase inhibitor (which was not reimbursed by national health insurance at that time). One hundred and thirty-eight patients (75.8%) were hormone-releasing hormone agonist with or without chemotherapy were not significantly different between the two groups, except older age was closely associated with the expression of MAP3K1 (p = 0.038).

Furthermore, we found that MAP3K1, p-ERK, p-JNK, and p-NF-κB expressions were downregulated in both the shMAP3K1 (#3)– and shMAP3K1 (#5)–transfected MCF7 group compared with the scrambled MCF7 group (Fig. 4F). In addition, the expression of MAP3K1, p-ERK, p-JNK, and p-NF-κB was decreased in tumor cells of both the shMAP3K1 (#3)–transfected T-47D group and the shMAP3K1 (#5)–transfected T-47D group compared with the scrambled T-47D group (Fig. 4F).

3.6 Expression of MAP3K1, p-ERK, and p-JNK is associated with poor clinical outcomes of patients with early-stage HR-positive, HER2-negative breast cancer

The median follow-up period for the patients was 12.0 years (95% confidence interval [CI]: 11.6–12.4); by the end of the follow-up period, 28 patients (15.4%) exhibited local recurrence and/or distant metastases, 17 patients (9.3%) had died (15 [88.2%] due to breast cancer and two [11.8%] due to causes not related to breast cancer), and 154 remained alive and healthy. The 10-year DFS and OS for all patients was 81.4% (95% CI: 78.2–87.6%) and 90.5% (95% CI: 86.2–94.8%), respectively.

The tumor stage (p = 0.812), tumor grade (p = 0.950), and axillary LN status (p = 0.968) were not associated with the
10-year DFS, whereas the LVI was marginally associated with the 10-year DFS (p = 0.099) (Table 2). Similarly, the tumor stage (p = 0.259), tumor grade (p = 0.876), and axillary LN status (p = 0.601) were not associated with the 10-year OS, whereas the LVI significantly correlated with the poor 10-year OS (p = 0.013) (Table 2). Furthermore, we found that patients with tumor cells expressing MAP3K1 exhibited a poor 10-year DFS than those without MAP3K1 expression (70.4% [95% CI: 58.1–82.7%] vs. 88.6% [95% CI: 82.1–95.5%], p = 0.006) and a poor 10-year OS (82.7% [95% CI: 73.9–91.5%] vs. 95.8% [95% CI: 91.7–99.9%], p = 0.005) (Fig. 6C and D). The p-JNK expression also significantly correlated with a poor 10-year DFS (p-JNK-positive group vs. p-JNK-negative group; 69.3% [95% CI: 54.8–83.8%] vs. 87.5% [95% CI: 80.8–94.2%], p = 0.018) and a poor 10-year OS (84.0% [95% CI: 74.8–88.7%] vs. 94.1% [95% CI: 89.6–98.6%], p = 0.034) (Fig. 6E F).

In the multivariate analyses (Table 2), we found that MAP3K1 expression was still an independent poor prognostic factor for DFS (hazard ratio [HR] = 3.036, 95% CI = 1.441 to 6.394, p = 0.003) and that LVI was marginally associated with poor DFS (HR = 2.039, 95% CI = 0.961–4.281, p = 0.063). By contrast, tumor stage (p = 0.870), histological grade (p = 0.937), and axillary LN (p = 0.710) were not associated with DFS. Similarly, the expression of MAP3K1 (HR = 5.696, 95% CI = 1.850–17.54, p = 0.002)

### Table 1

Clinicopathological features between MAP3K1-negative and MAP3K1-positive groups of HER-positive, HER2-negative breast cancer

| Number | Total (N) | Negative (N) | Positive (N) | P-value |
|--------|----------|--------------|--------------|---------|
| Age    | 182      | 109 (59.9%)  | 73 (40.1%)   | 0.038†  |
| Median | 49       | 47.5         | 50           |         |
| Range  | 23–81    | 23–81        | 35–74        |         |
| T-stage|          |              |              |         |
| T1     | 78 (42.9%) | 48 (44.0%)   | 30 (41.1%)   | 0.694‡  |
| T2     | 104 (57.1%) | 61 (56.0%)   | 43 (58.9%)   |         |
| Grade  |          |              |              |         |
| 1      | 70 (38.5%) | 46 (42.2%)   | 24 (32.9%)   | 0.401§  |
| 2      | 91 (50.0%) | 50 (45.9%)   | 41 (56.2%)   |         |
| 3      | 21 (11.5%) | 13 (11.9%)   | 8 (11.1%)    |         |

Abbreviation: HR, hormone receptor; HER2, human epidermal growth factor receptor 2; LN, lymph node; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; CE, cyclophosphamide and epirubicin; CMF, cyclophosphamide, methotrexate, and fluorouracil; AC, anthracycline and cyclophosphamide; Taxanes, paclitaxel or docetaxel; p-ERK, phospho-extracellular signal-regulated kinase; p-JNK, phospho-jun amino-terminal kinases

†P values (2-sided) were calculated using the Student t test
‡P values (2-sided) were calculated using the x² test or the Fisher exact test
§P values (2-sided) were calculated using 1-way analysis of variance

[Fig. 6A–F]
Clinicopathological features, including age, tumor size, tumor grade, LVI, and adjuvant endocrine regimens, were not different between patients with and without events (n = 57) (Supplementary Table 1). We detected MAP3K1 expression in 32 (43.8%) of 73 patients, whereas p-ERK expression was found in tumor cells of 37 (50.7%) patients, and p-JNK expression was present in tumor cells of 43 (58.9%) patients. Furthermore, MAP3K1 expression was significantly associated with the expression of p-ERK (p < 0.001, Spearman correlation coefficient, R = 0.518) and p-JNK (p < 0.001, Spearman correlation coefficient, R = 0.510). In addition, p-ERK expression was significantly

and the pathological manifestation of LVI (HR = 3.530, 95% CI = 1.291–9.650, p = 0.014) were independent poor prognostic factors for OS, whereas tumor stage (p = 0.670), histological grade (p = 0.949), and axillary LN status (p = 0.979) did not affect OS (Table 2).

3.7 Correlation between the expression of MAP3K1, p-ERK, and p-JNK and clinical outcomes based on an independent validation cohort

In an independent validation cohort, 16 patients had events, including locoregional recurrence, distant metastases, and death from breast cancer or other etiologies. Clinicopathological features, including age, tumor size, tumor grade, LVI, and adjuvant endocrine regimens, were not different between patients with and without events (n = 57) (Supplementary Table 1). We detected MAP3K1 expression in 32 (43.8%) of 73 patients, whereas p-ERK expression was found in tumor cells of 37 (50.7%) patients, and p-JNK expression was present in tumor cells of 43 (58.9%) patients. Furthermore, MAP3K1 expression was significantly associated with the expression of p-ERK (p < 0.001, Spearman correlation coefficient, R = 0.518) and p-JNK (p < 0.001, Spearman correlation coefficient, R = 0.510). In addition, p-ERK expression was significantly

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Discussion

In the present study, we demonstrated that MAP3K1 plays an important role in the pathogenesis of HR-positive and HER2-negative breast cancer. We identified a close link between MAP3K1 expression and the expression of MAP3K1-regulated molecules, p-ERK, and p-JNK in tumor samples from the experimental cohort (T1-T2 and N0-1, adjuvant endocrine therapy with and without chemotherapy) and an independent validated cohort (T1-T2 and N0, adjuvant endocrine therapy alone). Importantly, the overexpression of each of MAP3K1, p-ERK, or p-JNK significantly correlated with poor DFS and poor OS for both the experimental cohort and the independent validation cohort of patients with early-stage HR-positive and HER2-negative breast cancer.

Growing evidence suggests that MAP3K1 participates in cell proliferation, invasion, and migration of human pancreatic cancer cell lines and the cell migration of ovarian cancer cell lines [41–43]. Cuevas et al. reported that in the polyoma middle T antigen-driving mammary gland tumor, knockdown of MAP3K1 delays the dissemination and metastases of tumor cells [44]. Rangaswami et al. also found that the activation of MAP3K1-dependent MMP-9 signaling contributes to the osteopontin-triggered tumor growth and pulmonary metastases of melanoma [45]. These results are supported by our current findings that shMAP3K1 down-regulated MMP-9 expression and attenuated the cellular associating with p-JNK expression (Spearman’s correlation coefficient, R = 0.528; p < 0.001).

After a median follow-up of 10.05 years (95% CI, 10.323–10.677), the 10-year DFS rate and 10-year OS rate for this independent cohort were 79.2% (95% CI: 69.8–88.6%) and 84.4% (95% CI: 78.0–92.8%), respectively. Patients with tumors expressing MAP3K1 had a poorer 10-year DFS rate than those without MAP3K1 expression (65.0% [95% CI: 48.1–81.9%] vs. 90.2% [95% CI: 81.2–99.2%], p = 0.010) (Fig. 7). Similarly, the expression of p-ERK was significantly associated with a poor 10-years DFS rate (p-ERK-positive group vs. p-ERK-negative group; 66.7% [95% CI: 51.2–82.2%] vs. 91.7% [95% CI: 82.7–100%], p = 0.017), and the expression of p-JNK was also correlated with a poor 10-year DFS rate (p-JNK-positive group vs. p-JNK-negative group; 69.2% [95% CI: 55.3–83.1%] vs. 93.3% [95% CI: 84.3–100%], p = 0.027) (Fig. 7). Furthermore, patients with the overexpression of MAP3K1 had a significantly poorer 10-year OS than those without MAP3K1 expression (73.4% [95% CI: 57.4–89.4%] vs. 92.7% [95% CI: 84.7–100%], p = 0.037) (Fig. 7). The overexpression of p-ERK was also significantly associated with a poor 10-year OS (73.6% [95% CI: 58.7–88.5%] vs. 94.4% [95% CI: 87.0–100%], p = 0.041) (Fig. 7). The overexpression of p-JNK was also significantly correlated with a poor 10-year OS (75.2% [95% CI: 61.7–88.7%] vs. 96.7% [95% CI: 90.2–100%], p = 0.041) (Fig. 7).

4 Discussion

In the present study, we demonstrated that MAP3K1 plays an important role in the pathogenesis of HR-positive and HER2-negative breast cancer. We identified a close link between MAP3K1 expression and the expression of MAP3K1-regulated molecules, p-ERK, and p-JNK in tumor samples from the experimental cohort (T1-T2 and N0-1, adjuvant endocrine therapy with and without chemotherapy) and an independent validated cohort (T1-T2 and N0, adjuvant endocrine therapy alone). Importantly, the overexpression of each of MAP3K1, p-ERK, or p-JNK significantly correlated with poor DFS and poor OS for both the experimental cohort and the independent validation cohort of patients with early-stage HR-positive and HER2-negative breast cancer.

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MAP3K1 expression is associated with progression and poor prognosis of hormone receptor-positive, HER2-negative breast cancer patients. Disease-free survival (DFS) for all patients associated with the expression of MAP3K1 (B). Overall survival (OS) for all patients associated with the expression of MAP3K1 (C). DFS for patients associated with the expression of p-ERK (D). OS for patients associated with the expression of p-JNK (E). DFS for all patients associated with the expression of p-JNK (F). OS for all patients associated with the expression of p-JNK (G). MAP3K1 promotes HR-positive, HER2-negative breast cancer cell proliferation, migration, invasion, and resistance to the drugs (docetaxel, doxorubicin, and tamoxifen) through activating ERK, JNK, and NF-xB signaling; and inhibition of MAP3K1 attenuates tumor growth, migration and invasion, and enhances drugs sensitivities of this subtype of breast cancer through downregulation of ERK, JNK, and NF-xB signaling, and thus contributes to the better survival of this subtype of breast cancer.

Our findings showing that shMAP3K1 caused the G2/M phase arrest in cells via downregulation of cyclin B1 further supported the results of Hu et al. that transfection with MAP3K1 small interfering RNA significantly enhances the paclitaxel-mediated cell proliferation inhibition via G2/M phase arrest and downregulation of cyclin B1 expression [33]. The G2/M phase arrest might be a potential underlying mechanism of the enhanced cytotoxicity of docetaxel and doxorubicin in MCF7 and T-47D cells transfected with shMAP3K1 [46, 47]. In addition to the cell cycle arrest, we demonstrated that shMAP3K1 transfection led to induction of apoptosis via downregulation of Bcl-2 in MCF7 cells, and Bcl-xL in both MCF7 and T-47D cells, suggesting that MAP3K1 participates in the regulation of apoptosis.

Cuevas et al. reported that overexpression of MAP3K1 can promote the migration of fibroblast cells via activation of MAP3K1-ERK signaling-regulated calpain-dependent proteolysis of adhesion molecules [48]. Besides, several studies show that G protein-coupled estrogen receptor 1 (GPER, also known as GPR30) mediates the survival pathways for ER-positive breast cancer cells by triggering EGFR-dependent ERK signaling [49, 50]. Zhao et al. found that in vitro conditions, miRNA-302 can sensitize MCF7 and MCF7/ADR breast cancer cell lines to doxorubicin treatment via downregulation of MAP3K1/ERK signaling pathway [51]. Liu et al. revealed that p-ERK expression correlates with a poor DFS ($p = 0.049$) of 256 patients with earlystage breast cancer who received anthracycline-based...
Fig. 7 Association between MAP3K1 expression, p-ERK expression, or p-JNK in tumor cells and survival based on an independent validation cohort. (A). Disease-free survival (DFS) for all patients associated with the expression of MAP3K1 (B). Overall survival (OS) for all patients associated with the expression of MAP3K1 (C). DFS for all patients associated with the expression of p-ERK (D). OS for all patients associated with the expression of p-ERK (E). DFS for all patients associated with the expression of p-JNK (F). OS for all patients associated with the expression of p-JNK.
adjuvant chemotherapy [52]. In this study, we found that shMAP3K1 downregulated p-ERK and sensitized MCF7 and T-47D cells to doxorubicin. In tumor samples, our results further showed that p-ERK expression significantly correlated with MAP3K1 expression and with the poor DFS and OS of patients. These findings suggest that MAP3K1/ERK-signaling might be involved in the pathogenesis of HR-positive, HER2-negative breast cancer.

In addition to ERK signaling pathway, MAP3K1 also activates the JNK signaling and promotes cell proliferations and cell migrations [53]. In the MCF7 cells-xenograft models, the expression level of p-JNK and p-Jun were increased in tamoxifen-resistant tumors when compared with tamoxifen-sensitive tumors [54]. Gutierrez et al. revealed that expression of p-ERK and p-p38 MAPK (the upstream molecule of JNK/Jun signaling) was upregulated in relapsed tumor samples of patients who had ER-positive, HER2-negative breast cancer and received adjuvant tamoxifen when compared with paired pre-treatment specimens of the same patients [55]. These findings are supported by our current results showing that inhibition of MAP3K1 downregulated the expression of p-JNK and p-p38 MAPK in both breast cancer cell lines, and the expression of p-JNK was significantly associated with MAP3K1 expression and the poor DFS and OS of patients with HR-positive, HER2-negative early-stage breast cancer.

Previous studies have suggested that MAP3K1 may phosphorylate and activate IκBα and IκBβ kinase complexes and thus activate NF-κB in response to extracellular cytokines and stress [56, 57]. Other studies have revealed that NF-κB is involved in the epithelial-mesenchymal transition and metastasis of breast cancer cells [58, 59]. Following the assessment of the role of NF-κB expression in breast tumor samples, one study showed that the frequency of p65 expression is higher in tumor cells of HER2 and basal-like subtypes compared to its expression in luminal A subtype cancer cells [60]. Another study, however, reported an association between ER and NF-κB expression, where the NF-κB expression correlates with higher tumor grade, stage III-IV, and lymph node metastasis [61]. Furthermore, Oida et al. reported that NF-κB participated in the tamoxifen resistance in breast cancer cell line [62]. Previous studies have suggested that BCL3 not only activates NF-κB signaling via interaction with nuclear NF-κB p50 but also regulates the transcription of NF-κB-dependent genes [63–65]. Our findings demonstrated that inhibition of MAP3K1 downregulated NF-κB signaling pathway and BCL3 expression, and thus, suppressed cell growth of both HR-positive, HER2-negative breast cancer cell lines. Besides, inactivated NF-κB signaling may increase the sensitivity to tamoxifen in MCF7 and T-47D cells via downregulation of MAP3K1. These findings indicated that MAP3K1/NF-κB signaling might promote cell proliferation, enhance drug resistance, and contribute to the poor prognosis of this subtype of HR-positive, HER2-negative breast cancer.

In a study aimed at identifying somatic copy number changes and mutations in the exons of protein-coding genes in 100 breast cancer tumors, Stephens et al. identified several new cancer-related genes, including AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOR1, SMARCD1, and TBX, which may be involved in the tumorigenesis of breast cancer [66]. Among these driver mutations, somatic mutations of MAP3K1 are observed in 6% of breast cancers, predominantly in ER-positive breast cancer [66]. Furthermore, during the analyses of molecular heterogeneity of primary breast cancers through comprehensive molecular portraits, the authors showed that mutations of MAP3K1 are enriched in the HR-positive and HER2-negative breast cancer [67]. Further assessment of the association between MAP3K1 mutation and overexpression of MAP3K1 in patients with HR-positive and HER2-negative breast cancer is merited.

In addition to the expression of MAP3K1 in MCF7 cells, Liu et al. reported the mRNA and protein expression of MEKK1 (MAP3K1) in two triple-negative breast cancer cell lines, in human MDA-MB-231 breast cancer cells, and another murine 4T1 breast cancer cells [68]. Furthermore, Liu et al. showed that downregulation of MAP3K1 by MAP3K1-targeting therapeutic artificial microRNA (amiRNA), attenuated the proliferation and inhibited the migration and invasion of murine 4T1 breast cancer cells [68]. Further study to explore the biological function of MAP3K1 expression in human triple-negative breast cancer cell lines and patients with triple-negative breast cancer are warranted.

Although the cut-off points for the positive expression of MAP3K1 have not been described in previous reports, previous reports defined the cut-off points for the positive expression of p-ERK and p-JNK, which are the downstream signaling molecules of MAP3K1, as the positive nuclear staining of p-ERK or p-JNK in ≥ 20% of tumor cells [30, 31]. Therefore, in the current study, the positive expression of MAP3K1 was defined as a moderate or strong immunostaining of MAP3K1 in ≥ 20% of nuclei of tumor cells. We found that in the experimental cohort, a positive MAP3K1 expression was detected in the tumor cells of 73 of 182 patients (40.1%), whereas MAP3K1 expression was found in 32 of 73 patients (43.8%) in an independent validation cohort. However, in the cohort of breast cancer patients obtained from the Human Protein Atlas, most immunostainings of MAP3K1 proteins were located in the cytoplasmic and membranous cells of tumor cells. These findings may explain why the frequency (41.2%) of positive MAP3K1 expression in our cohort was lower than nearly 100% of
positive MAP3K1 expression in cases from the Human Protein Atlas.

In addition to our findings, Wang et al. revealed that glioma patients with a high expression of MAP3K1 (mostly located in the nucleus) in tumor cells had worse OS than those with low MAP3K1 expression [69]. In their study, the high expression of MAP3K1 was defined as an immunohistochemical score > 5 on the basis of the percentage of positive cells for MAP3K1 (0, negative; 1, < 10% positive; 2, 11–50% positive; 3, 51–80% positive; and 4, > 80% positive) and the intensity staining for MAP3K1 (0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive) [69]. Wu et al. also showed that a high expression of long noncoding RNAs (transcribed RNA molecules with lengths > 200 nucleotides and without open reading frames of significant length [< 100 amino acids]) of MAP3K1-2 was significantly associated with the poor survival of patients with gastric cancer [70].

5 Conclusion

In summary, we demonstrated that MAP3K1 might act as an essential factor for promoting HR-positive, HER2-negative breast cancer cell proliferation, migration, invasion, and resistance to the drugs, and thus, for increasing local recurrences and metastases of this subtype of breast cancer (Fig. 6G). Further studies on the possible relationship between MAP3K1 signaling and anti-apoptosis related proteins, such as Bcl-2 and Bcl-xL, are needed. Nevertheless, our results further elucidated the expression profiles of MAP3K1 in both experimental cancer cell lines, orthotopic animal model, and clinical specimens, and thus, it may facilitate the development of novel MAP3K1-related therapeutic strategies for this subtype of HR-positive, HER2-negative breast cancer.

Abbreviations

MAP3K1 Mitogen-Activated Protein Kinase Kinase 1
HR Hormone receptor
HER2 Human epidermal growth factor receptor 2
IHC Immunohistochemistry
ER Estrogen receptor
PR Progesterone receptor
HER2 Human epidermal growth factor receptor 2
FISH Fluorescence in situ hybridization
GWAS Genome-wide association studies
SNP Single nucleotide polymorphisms
MAP3K1 Mitogen-Activated Protein Kinase Kinase 1
DFS Disease-free survival
OS Overall survival
JNK Jun amino-terminal kinases
ERK Extracellular-signal-regulated kinase
MKP-1 MAPK phosphatase-1
p Phospho
shRNA Short hairpin RNA
RT-qPCR Real-time quantitative polymerase chain reaction
SD Standard deviation
PBS Phosphate-buffered saline
FACS Fluorescence-activated cell sorting
SDS Sodium dodecyl sulfate
PAGE Polyacrylamide gel electrophoresis
PVDF Polyvinylidene difluoride
MMP Matrix metalloproteinase
LVI Lymphovascular invasion
LN Lymph node
GPER G protein-coupled estrogen receptor 1

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Authors’ contributions SHK designed the study and participated in the data acquisition, analysis, and interpretation, and writing and reviewing of the manuscript. MFW, JCL, and WCY performed the laboratory work. YHL participated in the pathologic review and immunohistochemical data interpretation. SYY performed the statistical analysis. CSH participated in the data acquisition, analysis, and interpretation, writing, and reviewing of the manuscript, administrative, technical, and material support. All authors contributed to the interpretation of the results and critically reviewed the draft of the manuscript, read and approved the final version of the manuscript.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval and Consent to participate (Human and animal ethics statements) The immunohistochemical analyses of protein expression of MAP3K1, p-ERK, and p-JNK and detailed demographic information were obtained from the patients and their medical charts with their written informed consent. The pathologic review and immunohistochemical studies were approved by the National Taiwan University
Hospital (NTUH) ethics committee (Institutional Review Board [IRB] Number: 201804056RINB). The patients’ medical data were anonymized before access and analysis. All animal experimental procedures were revised and approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee (IACUC; Number 20170503).

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