MAP3K1 Expression is Associated With Progression and Poor Prognosis of Hormone Receptor-Positive, HER2-Negative Early-Stage Breast Cancer

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Abstract

Background: MAP3K1 (Mitogen-Activated Protein Kinase Kinase Kinase 1) participates in the MAPK signal transduction pathway, responding to a number of mitogenic and metabolic stimuli, which may be related to breast cancer susceptibility and progression. In this study, we assessed whether overexpression of MAP3K1 promotes proliferation, migration, and invasion of breast cancer cells, and thus, affects 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*), both overexpressing MAP3K1, were transfected with MAP3K1 short hairpin RNA plasmids (shMAP3K1) and their proliferation, migration, and invasion were examined. We assessed whether shMAP3K1 affects the cell cycle and levels of downstream signaling molecules, such as ERK and NF-κB, as well as sensitizes cells in both cell lines to chemotherapeutic and hormonal agents. A total of 161 patients with HR-positive, HER2-negative T1-T2 breast cancer and 0 to 3 nodal metastases were included. The expression of MAP3K1 and phospho (p)-ERK proteins was assessed by immunohistochemistry.

Results: In both cell lines (MCF7 and *T-47D*), shMAP3K1 significantly reduced cell growth, migration, and invasion by downregulating MMP-9 and blocking the G2/M phase of the cell cycle and its regulatory molecule cyclin B1. Besides, shMAP3K1 downregulated ERK- and NF-κB-dependent gene transcription, and enhanced sensitivities of both cell lines to doxorubicin, docetaxel, and tamoxifen. Patients with MAP3K1 overexpression exhibited significantly poor 9-year disease-free survival (DFS; 72.6% vs. 88.5%, $p = 0.022$) and overall survival (OS; 83.8% vs. 96.2%, $p = 0.012$) relative to those without MAP3K1 overexpression. Furthermore, the p-ERK expression was significantly associated with MAP3K1 expression ($p < 0.001$) and correlated with a poor 9-year DFS ($p = 0.033$) and OS ($p = 0.023$).

Conclusions: Our results indicate that overexpression of MAP3K1 plays a major role in poor prognosis of HR-positive, HER2-negative early-stage breast cancer.

Background

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 (LNs), whereas endocrine treatments are dispensed for those without high-risk factors [6–9].

In addition to clinicopathological features, several multiple 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 recently 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 JNK1/2 (also known as MAPK8/9), extracellular-signal-regulated kinase (ERK)1/2, or 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].

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 [23]. Among these driver mutations, somatic mutations of MAP3K1 are observed in 6% of breast cancers, predominantly in ER-positive breast cancer [23]. 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 [24].

Based on aforementioned evidences [19–24], 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 and NF-κB activity in the *in vitro* HR-positive, HER2-negative breast cancer cell lines. 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, p-ERK, correlated with the expression of MAP3K1 and the clinical outcome of the same group of tumors.

Materials And Methods

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% CO_2 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 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 lentivirus 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 shMAP3K1 using RNeasy Mini Kit (*#74106*,Qiagen, Hilden, Germany). Reverse transcription reaction was performed using the Maxima first strand cDNA Synthesis Kit for real-time quantitative PCR (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'-GTTTCCTCAGGGCTATATGGTG-3' (reverse). The reactions were performed in triplicates.

Cell proliferation, viability, migration, and invasion assay

The detailed information of cell proliferation, viability, migration, and invasion assay are listed in the **Supplementary methods** [25].

Cell cycle analysis, apotposis analysis, luciferase assay, and immunoblotting analysis

The detailed information of cell cycle analysis, apoptosis analysis, luciferase assay, and immunoblotting assay are listed in the **Supplementary methods** [26-30].

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 ≥ 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 ≥ 2.0, tumors were considered positive based on the *American Society of Clinical Oncology* guidelines [31]. 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, and LN positivity (N1), received standard adjuvant chemotherapy, such as CMF (cyclophosphamide, methotrexate, fluorouracil), CEF (cyclophosphamide, epirubin, 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 hormonal 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.

Immunohistochemistry analysis

The immunohistochemistry staining for MAP3K1 (clone 256, Abgent, San Diego, CA, USA) [28] and p-ERK [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 and/or cytoplasm) 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, 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, positive expression was defined as positive nuclear staining in 20% of tumor cells (20–40%, score = 2; >40%, score = 3), whereas p-ERK-negative (score = 0), focally positive tumor, or tumor cells with positive p-ERK staining <20% (score = 1) was defined as negative, as previously described [32].

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 and p-ERK was analyzed by Spearman's correlation.

For breast cancer patients, follow-up data available on December 31, 2011, 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 [33]. The DFS and OS were calculated using the Kaplan–Meier method, and the survival curves were compared using the log-rank test. The clinical characteristics were compared using the chi-square test and Fisher's exact tests. $P < 0.05$ was considered statistically significant.

Results

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

As shown in **Supplemental Fig. S1** (shMAP3K1-targeted mRNA sequences) and **Supplemental Fig. S2 (**shMAP3K1-targeted amino acid sequences), we assessed the efficiencies of shMAP3K1-mediated inhibition in both MCF7 and T-47D cells. For that, we assessed mRNA levels of MAP3K1 in control MCF7 and T-47D cells, and the cells transfected with shMAP3K1 using RT-qPCR. We found that mRNA expression levels of MAP3K1 were significantly downregulated in shMAP3K1-transfected MCF7 cells and shMAP3K1-transfected T-47D cells when compared with scrambled MCF7 cells and scrambled T-47D cells, respectively (**Supplemental 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-transfected MCF7 cells and in

shMAP3K1-transfected T-47D cells (combination of primary and secondary antibodies). The results are illustrated in **Supplemental Fig. S3**.

These breast cancer cell lines expressing higher levels of MAP3K1 were transfected with shMAP3K1 to downregulate the MAP3K1 protein expression (**Fig. 1a**). When compared with the scrambled group, shMAP3K treatment significantly reduced the expression level of MAP3K1 by 84% in MCF7 cells ($p < 0.001$), and by 75% in T-47D cells ($p < 0.001$) (**Fig. 1a**). Proliferation assay showed that the downregulation of MAP3K1 decreased cell number in both breast cancer cell lines (**Fig. 1b**). The cell number was significantly inhibited at Day 3, Day 5, and Day 7 of shMAP3K1-transfected MCF7 and T-47D cells. The results of the migration assay showed that the number of migrated cells was reduced by 48.1% in shMAP3K1-transfected MCF7 and by 82.3% in shMAP3K1-transfected T-47D cells when compared with scramble-transfected MCF7 and T-47D cells (**Fig. 1c**). The results of the invasion assay showed that the number of invaded cells was reduced by 73.7% in MCF7 cells and by 63.1% in T-47D cells when compared with scrambled MCF7 and T-47D cells (**Fig. 1d**). These findings indicated that inhibition of MAP3K1 suppressed cell number, cell migration, and cell invasion in these two HR-positive, HER2-negative breast cancer cell lines.

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-12F cells (normal mammary epithelial cell line) [34]. 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-transfected MCF7 cells, there was a significant increase in the number of cells in the G2/M phase of cell cycle, and concomitantly, a significant decrease in the number of cells in the G0/G1 phase was observed. On the contrary, in T-47D cells, shMAP3K1 treatment led to an arrest of a significant number of cells in the G2/M phase of cell cycle, and there was a significant decrease in the number of cells in the S phase. These findings indicated that shMAP3K1 blocked cell cycle progression through the G2/M phase. *Annexin V* staining revealed that shMAP3K1 treatment resulted in increased apoptotic events (early and late apoptosis level) in both MCF7 cells (16.34% ± 0.77% vs. 1.84 % ± 0.42%, $p = 0.000633$) and T-47D cells (13.68% ± 0.31% vs. 4.87% ± 0.16%, $p = 0.0000517$) when compared with scrambled MCF7 and T-47D cells, respectively (**Fig. 2b**).

The shMAP3K1 resulted in the cell cycle arrest at the G2/M phase, which indicated that the downregulation of MAP3K1 might increase drug sensitivity. As shown in **Fig. 2c**, we found that inhibition of MAP3K1 promoted decrease in the cell viability in tamoxifen-treated MCF7 (at 0.01, 0.1, 1, 10, and 50 μM) and T-47D (at 0.01, 0.1, 1, 10, and 100 μM) cells when compared with cells transfected with scrambled shRNA. *Furthermore, inhibition of MAP3K1 increased the cellular drug sensitivity for*

*doxorubicin (at 0.01, 0.1, 1, and 2 μM) in MCF7 cells, for doxorubicin (at 0.01, 0.1, 1, 10, and 50 μM) in T47-D cells, and for docetaxel (at 0.01, 0.1, 1, 10, and 20 μM) in both MCF7 and T-47D cells (**Fig. 2d and Fig. 2e**).*

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

We found that the expression of cyclin B1 was essentially reduced by shMAP3K1 transfection, while the expression of cyclin D1 (key molecule for *G1 arrest* during the cell cycle) was not affected (**Fig. 3a**). Considering that ERK1/2 is a downstream molecule of MAP3K1-signaling pathway [20], we assessed whether ERK1/ERK2 can be inhibited by *MAP3K1 silencing. We found that shMAP3K1 transfection* downregulated *p-ERK* expression by 83% in MCF7 cells and by 42% in T-47D cells *when compared with the scrambled group (**Fig. 3a**).*

Previous studies have revealed that the deletion of MAP3K1 results in apoptosis when mouse embryonic stem cells are subjected to hyperosmolarity and microtubule disruption or cardiomyocytes are subjected to oxidative stress [35, 36]. Zang et al. reported that MAP3K1 silencing inhibits cell proliferation and increases apoptosis of esophageal squamous cell carcinoma cells [37]. However, the role of MAP3K1 in regulating anti-apoptotic function in breast cancer cells remains unclear. The members of Bcl-2-related anti-apoptotic protein family, including Bcl-2 and Bcl-xL, play critical roles in the pathogenesis of ER-positive breast cancer cells [38-40]. Therefore, we assessed whether shMAP3K1 transfection led to the downregulation of expression of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in both MCF7 and T-47D cells. We found that shMAP3K1 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 also led to increased expression of cleaved poly (ADP-ribose) polymerase (PARP) and decreased matrix metalloproteinase (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, acting as a downstream factor in the MAP3K1 signaling pathway, is involved in the pathogenesis of HR-positive breast cancer cells [41]. As shown in **Fig. 3c**, transfection with shMAP3K1 inhibited the expression of p-IκBα, an essential regulatory molecule of NF-κB, in both breast cancer cell lines. Also, transfection with shMAP3K1 downregulated the nuclear expression of NF-κB (p65) in both breast cancer cell lines. Furthermore, NF-κB-dependent expression of genes, such as nuclear p52 and BCL3, was downregulated in MCF7 and T-47D cells after transfection with shMAP3K1 (**Fig. 3c**). 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 (**Fig. 3d**).

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.

Expression of MAP3K1 and p-ERK in tumor cells of patients with early-stage HR-positive, HER2-negativebreast 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 161 patients with either T1 or T2 status and negative or 1 to 3 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 hormonal therapy; sixteen patients (9.9%) received ovarian ablation or a luteinizing hormone-releasing hormone agonist with or without tamoxifen, and 145 (90.1%) 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 twenty-two patients (75.8%) were LN-negative, whereas 39 patients (24.2%) had 1 to 3 LN metastases. Seventy-four patients (46.0%) did not receive any chemotherapy, and 87 patients (54.0%) received standard adjuvant chemotherapy (**Table 1**). Furthermore, 121 patients (75.2 %) were positive for both ER and PR.

We detected MAP3K1 expression (51 cases, score 2; 13 cases, score 3) in tumor cells of 64 patients (39.8%), whereas 97 patients exhibited negative MAP3K1 expression (92 cases, score 0; five cases, score 1) (**Fig. 4a-c**). **Table 1** displays the demographic characteristics of the two groups of patients (MAP3K1-positive vs. MAP3K1-negative) and their clinicopathological features. Age, tumor size, histological grade, axillary LN, ER and PR status, and adjuvant chemotherapy were not significantly different between the two groups.

Considering that ERK1/2 is a downstream molecule of MAP3K1-signaling pathway [20], we assessed whether the expression of p-ERK correlates with the expression of MAP3K1 in the same group of patients. In the selected 145 patients who had available tumor samples, we detected MAP3K1 expression (52 cases, score 2; 9 cases, score 3) in tumor cells of 61 patients (42.0%), whereas 84 patients exhibited negative MAP3K1 expression (73 cases, score 0; 11 cases, score 1) (**Fig. 4d-f)**. The p-ERK expression was more frequently detected in MAP3K1-positive tumors (42/59 [71.2%]) than in MAP3K1-negative tumors (19/86 [22.1%]) of patients ($p < 0.001$, **Fig. 4e** and **Table 1**).

Expression of MAP3K1 or p-ERK 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 8.97 years (95% confidence interval [CI]: 8.57 to 9.37); by the end of the follow-up period, 25 patients (15.5%) exhibited local recurrence and/or distant metastases, 12 patients (7.5%) had died (11 [91.7%] due to breast cancer and one [8.3%] due to causes not related to breast cancer), and 149 remained alive and healthy. The 9-year DFS and OS for all patients was 82.2% (95% CI: 75.3% to 89.1%) and 91.0% (95% CI: 85.9% to 96.1%), respectively.

The tumor stage (p = 0.838), tumor grade (p = 0.553), and axillary LN status (p = 0.763) were not associated with the 9-year DFS (**Table 2**). Similarly, the tumor stage (p = 0.551), tumor grade (p = 0.679), and axillary LN status (p = 0.943) were not associated with the 9-year OS (**Table 2**). Furthermore, we found that patients with tumor cells expressing MAP3K1 exhibited a poor 9-year DFS than those without MAP3K1 expression (72.6% [95% CI: 59.5% to 85.7%] vs. 88.5% [95% CI: 81.6% to 95.4%], p = 0.022) (**Fig. 5a**). Similarly, overexpression of MAP3K1 was significantly associated with poor 9-year OS (MAP3K1-positive group vs. MAP3K1-negative group, 83.8% [95% CI: 73.6% to 94.0%] vs. 96.2% [95% CI: 91.9% to 100%], p = 0.012) (**Fig. 5b**). Furthermore, p-ERK expression significantly correlated with a poor 9-year DFS (p-ERK-positive group vs. p-ERK-negative group; 74.6% [95% CI: 62.3% to 86.9%] vs. 87.3% [95% CI: 79.9% to 94.7%], p = 0.033) and a poor 9-year OS (86.4% [95% CI: 78.2% to 94.6%] vs. 95.9% [95% CI: 91.2% to 100%], p = 0.023) (**Fig. 5c-d**).

Using multivariate analyses (**Table 2**), we found that MAP3K1 expression was still an independent prognostic factor for DFS (hazard ratio = 2.476, 95% CI = 1.112 to 5.517, p = 0.026), whereas tumor stage (p = 0.769), histological grade (p = 0.554), and axillary LN (p = 0.582) were not associated with DFS. Similarly, MAP3K1 expression was still an independent prognostic factor for OS (hazard ratio = 4.489, 95% CI = 1.214 to 16.604, p = 0.024), whereas tumor stage (p = 0.629), histological grade (p = 0.755), and axillary LN status (p = 0.983) did not affect OS (**Table 2**).

Discussion

In the present study, we demonstrated that MAP3K1 plays an important role in the pathogenesis of HR-positive, HER2-negative breast cancer cells. This conclusion was based on the following findings from our study: 1) the inhibition of MAP3K1 by shMAP3K1 efficiently inhibited the cell number of breast cancer cells by blocking cell cycle progression in the G2/M phase, inhibiting the G2/M phase-related cell cycle protein, cyclin B1, and by downregulating anti-apoptosis-related molecules, Bcl-2 and Bcl-xL, in MCF7 and T-47D cells, respectively; 2) inhibition of MAP3K1 attenuated the migration and the invasion of breast cancer cells by downregulating MMP-9 and p-ERK; 3) inhibition of MAP3K1 resulted in downregulation of expression of p-IκBα, downregulation of NF-κB-associated genes (including p65 and p52), and decrease in the luciferase activity of NF-κB; 4) inhibition of MAP3K1 sensitized breast cancer cells to chemotherapy and anti-estrogen agents; 5) overexpression of MAP3K1 in tumor cells was significantly associated with the poor DFS and OS of patients with early-stage HR-positive, HER2-negative breast cancer; 6) Expression of p-ERK in tumor cells significantly correlated with the expression of MAP3K1 and poor DFS and OS of the same group of patients.

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 [42–44]. 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 [45]. 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 [46]. These results are supported by our current findings that

shMAP3K1 downregulated MMP-9 expression and attenuated the cellular survival, migration, and invasion in both breast cancer cell lines.

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 [34]. The G2/M phase arrest might be a potential underlying mechanism of the enhanced cytotoxicity of docetaxel [47] and doxorubicin [48] in MCF7 and T-47D cells transfected with shMAP3K1. 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-ERK1/2 signaling-regulated calpain-dependent proteolysis of adhesion molecules [49]. 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 ERK1/2 signaling [50, 51]. Zhao et al. found that in *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 [52]. 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 anthracyclinebased adjuvant chemotherapy [53]. 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.

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 [54, 55]. Other studies have revealed that NF-κB is involved in the epithelial-mesenchymal transition and metastasis of breast cancer cells [56, 57]. 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 [58]. 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 [59]. Furthermore, Oida et al. reported that NF-κB participated in the tamoxifen resistance in breast cancer cell line [60]. 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 [61–63]. 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 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 [64]. Furthermore, Liu et al. showed that downregulation of MAP3K1 by MAP3K1targeting therapeutic artificial microRNA (amiRNA), attenuated the proliferation and inhibited the migration and invasion of murine 4T1 breast cancer cells [64]. In the xenograft model of BALB/c-nude mice, MAP3K1 amiRNA suppresses tumor growth and decreases lung metastasis of murine 4T1 breast cancer cells [64]. These findings indicate that MAP3K1 may play a role in the development and progression of triple-negative breast cancer cells. 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.

Conclusion

In summary, we demonstrated that MAP3K1 acts via activation of MMP-9, ERK, and NF-κB in *in vitro* HR-positive, HER2-negative breast cancer cell lines, and that overexpression of MAP3K1 is significantly associated with poor DFS and OS of HR-positive, HER2-negative early-stage breast cancer patients. We also revealed that MAP3K1 signaling contributes to the resistance to chemotherapeutic drugs and anti-estrogen agents. These findings suggested 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. 5e). 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 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 Kinase 1; HR, hormone receptor; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; ER: estrogen receptor; PR: progesterone receptor (PR): FISH: fluorescence in situ hybridization; GWAS; genome-wide association studies; SNP: single nucleotide polymorphisms; DFS: disease-free survival; OS: overall survival; ERK: extracellular-signal-regulated kinase; LN: lymph node; p: phospho; SD, standard deviation.

Declarations

Acknowledgments:

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Author Contributions:

SHK designed the study and participated in the data acquisition, analysis, and interpretation, and writing and reviewing of the manuscript. MFW, WCY, JCL. 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

All data presented and analyzed during this study are included in this article.

Ethics approval and consent to participate

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 (strict compliance to the Declaration of Helsinki.). 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.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interests.

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Tables

Table 1
Clinicopathological features between MAP3K1-negative and MAP3K1-positive groups of HR-positive, HER2-negative breast cancer

		MAP3K1		
	Total (N)	**Negative**	**Positive**	**P-value**
Number	161	97 (60.2%)	64 (39.8%)	
Age				0.130†
Median	49	47.5	50	
Range	23–81	23–81	35–74	
T-stage				0.691‡
T1	71 (44.1%)	44 (45.4%)	27 (42.2%)	
T2	90 (55.9%)	53 (54.6%)	37 (57.8%)	
Grade				0.390§
1	62 (38.5%)	41 (42.3%)	21 (32.8%)	
2	81 (50.3%)	45 (46.4%)	36 (56.2%)	
3	18 (11.2%)	11 (11.3%)	7 (11.0%)	
Axillary LN				0.852‡
Negative	122 (75.8%)	74 (76.7%)	48 (75.0%)	
Positive	39 (24.2%)	23 (23.3%)	16 (25.0%)	
ER and PR status				0.407§
ER (+)PR(+)	121 (75.2%)	75 (77.3%)	46 (71.8%)	
ER(+)/PR(-)	21 (13.0%)	12 (12.4%)	9 (14.1%)	
ER(-)/PR(+)	19 (11.8%)	10 (10.3%)	9 (14.1%)	
Chemotherapy				0.206‡

Abbreviation: HR, hormone receptor; HER2, human epidermal growth factor receptor 2; LN, lymph node; ER, estrogen receptor; PR, progesterone receptor; CE, cyclophosphamide and epirubicin; CMF, cyclophosphamide, methotrexate, and fluorouracil; AC, anthracycline and cyclophosphamide; Taxanes, paclitaxel or docetaxel; p-EGFR, phospho-extracellular signal-regulated kinase.

†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.

		MAP3K1		
No	74 (46.0%)	47 (48.5%)	27 (42.2%)	
CE	45 (28.0%)	29 (29.9%)	16 (25.0%)	
CMF	32 (19.9%)	15 (15.5%)	17 (26.6%)	
AC	7 (4.2%)	5 (5.1%)	2 (3.1%)	
Taxanes	3 (1.9%)	1 (1.0%)	2 (3.1%)	
p-ERK expression (N = 145)				< 0.001
Negative	84 (57.9%)	67 (77.9%)	17 (28.8%)	
Positive	61 (42.1%)	19 (22.1%)	42 (71.2%)	

Abbreviation: HR, hormone receptor; HER2, human epidermal growth factor receptor 2; LN, lymph node; ER, estrogen receptor; PR, progesterone receptor; CE, cyclophosphamide and epirubicin; CMF, cyclophosphamide, methotrexate, and fluorouracil; AC, anthracycline and cyclophosphamide; Taxanes, paclitaxel or docetaxel; p-EGFR, phospho-extracellular signal-regulated kinase.

†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.

Table 2
Univariate and multivariate analyses of the relationship between pathological features and expression of MAP3K1 and clinical outcomes of HR-positive and HER2-negative breast cancer patients

	Univariate analyses			Multivariate analyses		
DFS	HR*	95% CI	*P* value	HR*	95% CI	*P* value
T-stage T2 vs. T1	0.921	0.418–2.031	0.838	0.887	0.401–1.966	0.769
Grade III vs. I + II	1.176	0.689–2.007	0.553	1.176	0.688–2.009	0.554
LN Pos vs. Neg	0.763	0.286–2.033	0.763	0.758	0.283–2.030	0.582
MAP3K1 Pos vs. Neg	2.465	1.107–5.488	0.027	2.476	1.112–5.517	0.026
OS	HR*	95% CI	*P* value	HR*	95% CI	*P* value
T-stage T2 vs. T1	1.142	0.433–4.795	0.551	1.348	0.402–4.514	0.629
Grade III vs. I + II	1.174	0.549–2.508	0.679	1.129	0.526–2.423	0.755
LN Pos vs. Neg	1.049	0.284–3.875	0.943	0.986	0.263–3.699	0.983
MAP3K1 Pos vs. Neg	4.559	1.234*16.848	0.023	4.489	1.214–16.604	0.024

Abbreviations: MAP3K1, mitogen-activated protein kinase kinase kinase 1; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; DFS, disease-free survival; HR*, hazard ratio; CI, confidence interval; T, tumor; LN, lymph node; Pos, positive; Neg, negative; OS, overall survival.

Figures

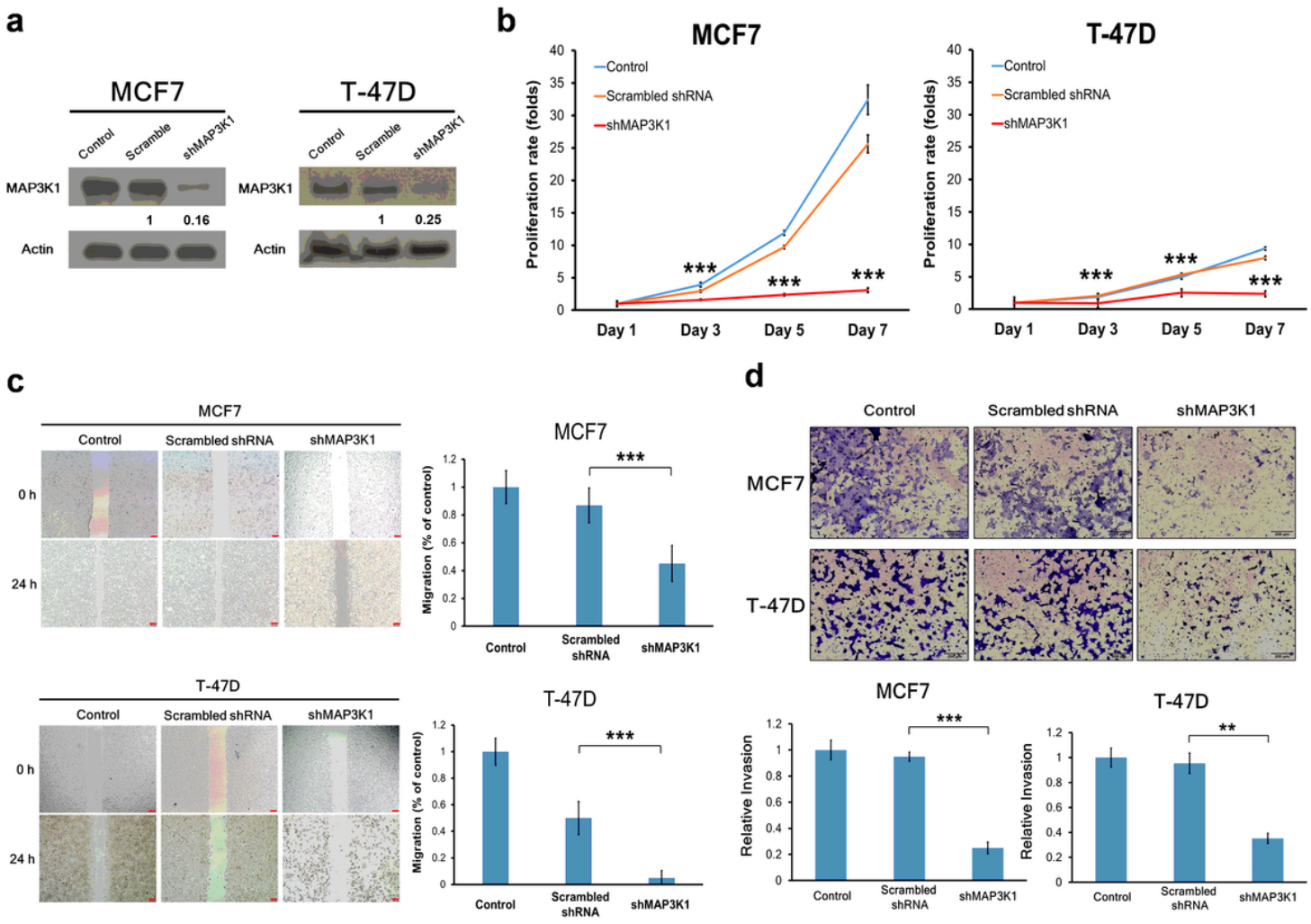

Figure 1

Figure 1

Silencing of the MAP3K1 gene inhibits cellular proliferation and migration in HR-positive, HER2-negative breast cancer cell lines. a Two breast cancer cell lines, MCF7 and T-47D, were infected with pGIPZ lentiviral (scrambled) and MAP3K1 shRNA lentiviral vectors. The protein expression of MAP3K1 was examined by western blotting analysis. The data showed an efficient knockdown of MAP3K1 in breast cancer cell lines transfected with shMAP3K1, and therefore, we used this shRNA to conduct further experiments. b Proliferation assay showed that the MAP3K1-silenced cell line exhibited a significant reduction in cell number compared to the scrambled group of the same cell line. c Cell migration assay showed that MAP3K1 knockdown inhibited breast cancer cell migration at 24 hours (no differences between scrambled- and shMAP3K1-transfected breast cancer cell lines). Data are shown as mean ± SD from three independent experiments. d Invasion assay showed that MAP3K1 knockdown suppressed breast cancer cell invasion in both shMAP3K1-transfected MCF7 and T-47D cells when compared with scrambled MCF7 and T-47D cells. The invaded cells were stained with crystal violet and counted in four different microscopic fields. Data are shown as mean ± SD from three independent experiments. **, p <

0.01; ***, p < 0.001. MAP3K1, mitogen-activated protein kinase kinase kinase 1; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; shRNA, short hairpin RNA; SD, standard deviation.

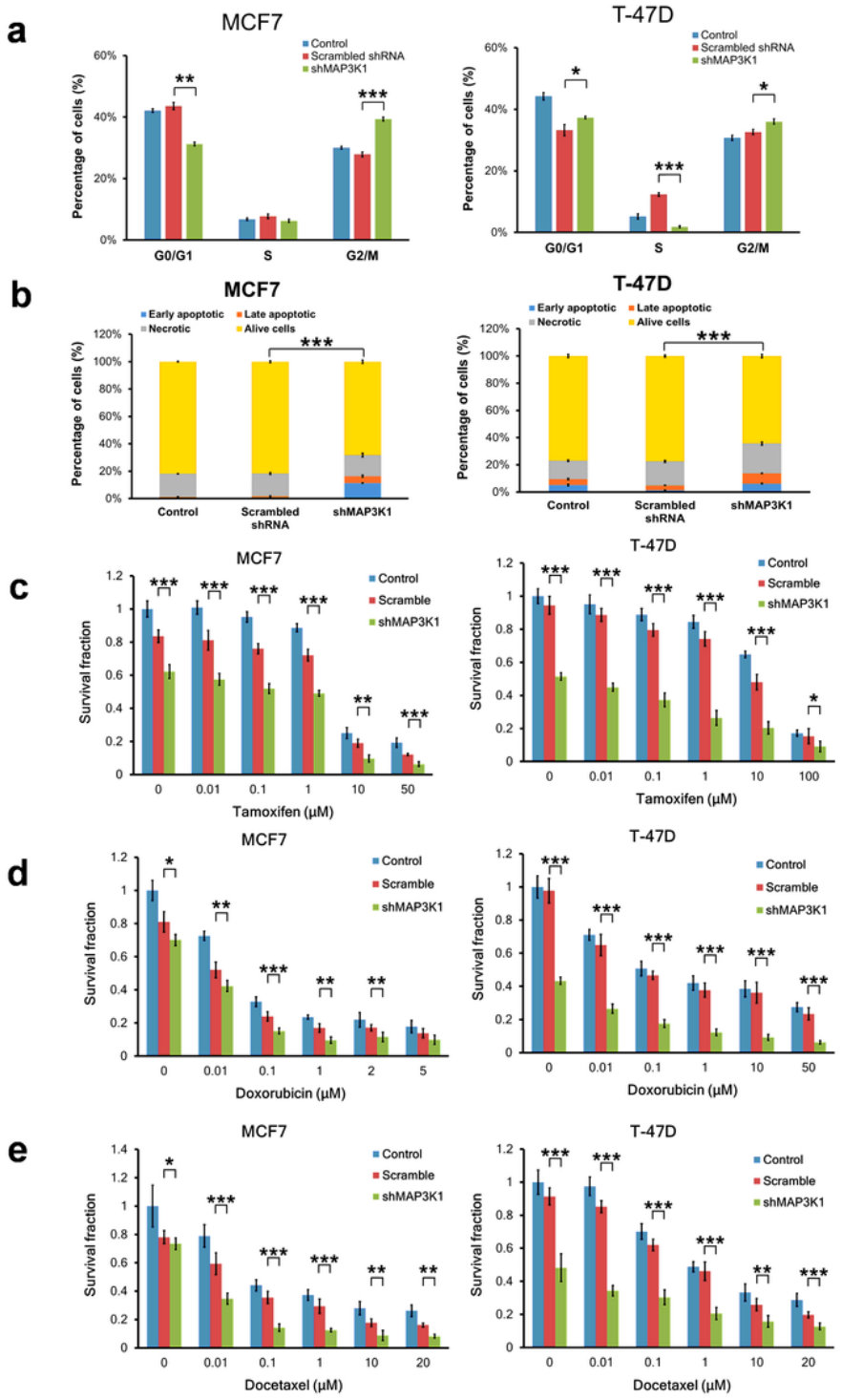

Figure 2

Figure 2

Inhibition of MAP3K1 causes G2/M phase arrest and increases drug sensitivity in HR-positive, HER2-negative breast cancer cell lines. a shMAP3K1 transfection resulted in G2/M phase arrest in both MCF-7 and T-47D cells. The results are expressed as the mean ± SD of three independent experiments from each

cell line. b Summary of the Annexin V-FITC apoptosis assay results showing the percentages of early apoptotic, late apoptotic, necrotic, and living cells. The results are expressed as the mean ± SD of three independent experiments from each cell line. Annexin V staining revealed that shMAP3K1 treatment resulted in more prominent apoptosis in both MCF7 and T-47D cells. c shMAP3K1 promoted inhibition of cell viability in tamoxifen-treated MCF7 and T-47D cells when compared with scrambled groups. d shMAP3K1 promoted inhibition of cell viability in doxorubicin-treated MCF7 and T-47D cells when compared to the scrambled groups. e shMAP3K1 increased the sensitivity to docetaxel in both MCF7 and T-47D breast cancer cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. MAP3K1, mitogen-activated protein kinase kinase kinase 1; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; shRNA, short hairpin RNA; SD, standard deviation.

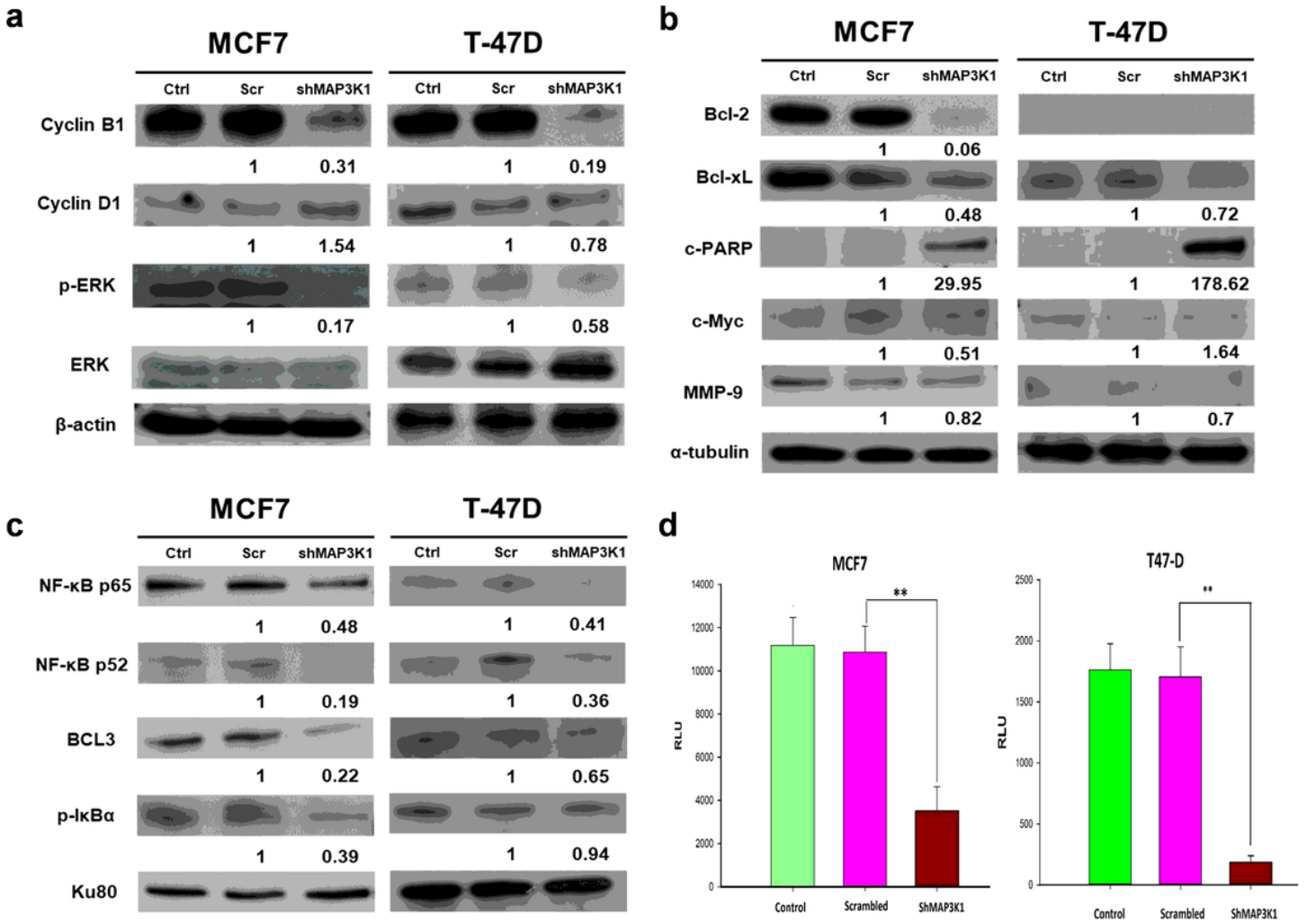

Figure 3

Figure 3

Inhibition of MAP3K1 downregulates cell cycle-, anti-apoptosis-, and MAP3K1-related proteins and decreases the NF-κB-dependent gene transcription. a shMAP3K1 transfection downregulated the expression of cyclin B1 and p-ERK; however, it did not affect cyclin D1 in total cell lysates of both MCF-7 and T-47D cancer cells. b shMAP3K1 transfection downregulated the expression of Bcl-2, Bcl-xL, and

MMP-9, and increased the levels of c-PARP; however, it did not affect c-Myc expression in total cell lysates of MCF7 cells. shMAP3K1 transfection decreased the expression of Bcl-xL and MMP-9, and increased the c-PARP levels; however, it did not affect c-Myc expression in total cell lysates of T-47D cells. c shMAP3K1 decreased the expression of p65, p52, and BCL3 in nuclear lysates, and p-IκBα in total lysates of both MCF7 and T-47D breast cancer cells. d The results from three independent experiments using shMAP3K1-transfected MCF7 and T-47D cells are presented as RLU per milligram of protein (NF-κB-Luc promoter activity-luciferase assay) (**, $p < 0.01$). MAP3K1, mitogen-activated protein kinase kinase kinase 1; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; shRNA, short hairpin RNA; p-ERK, phospho-extracellular signal-regulated kinase; MMP-9, matrix metalloproteinase-9; c-PARP, cleaved poly (ADP-ribose) polymerase; RLU, relative luciferase units.

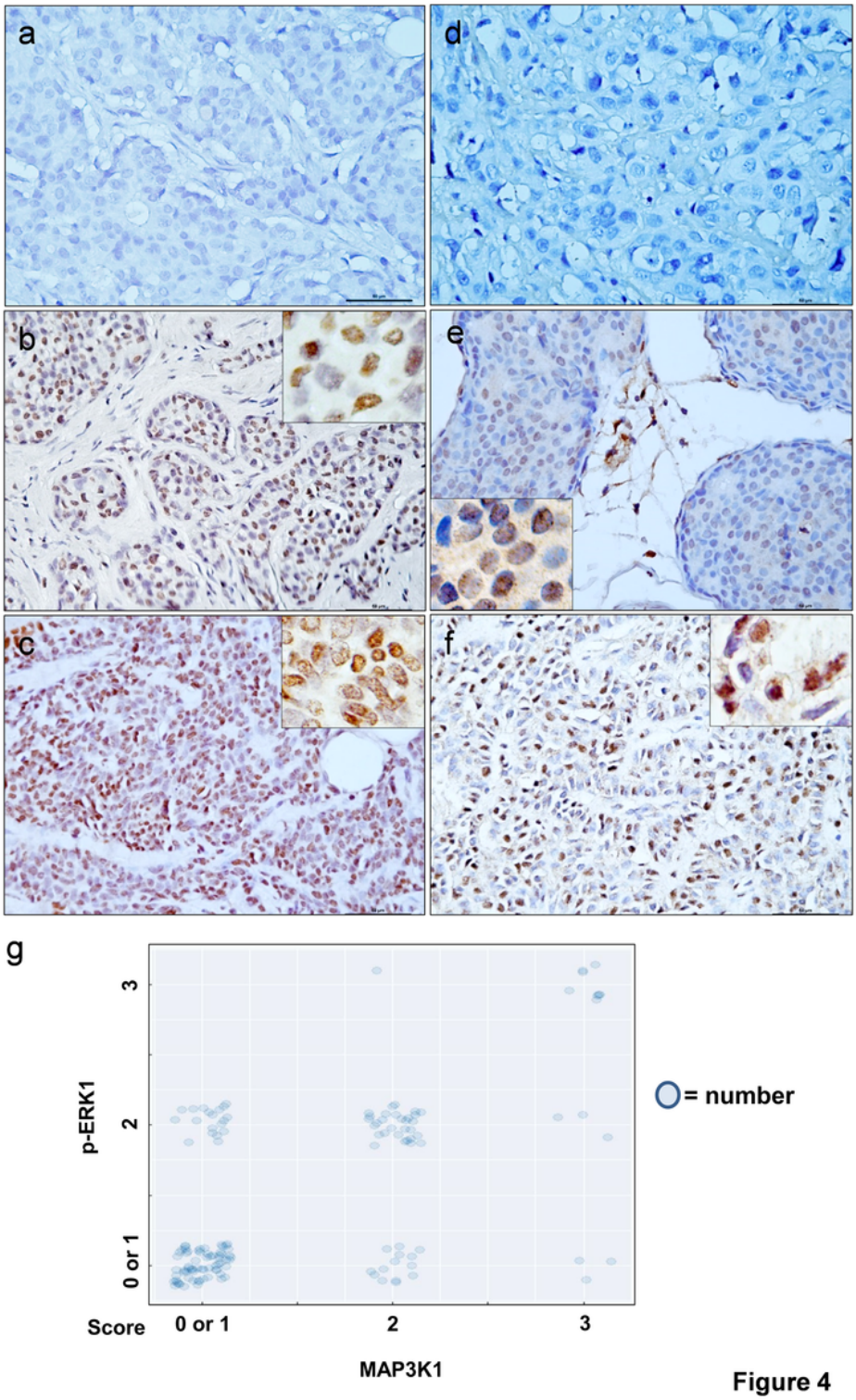

Figure 4

Figure 4

Expression of MAP3K1 and p-ERK in tumor cells of early-stage HR-positive, HER2-negative breast cancer patients. a Negative expression of MAP3K1 in tumor specimens of breast IDC. b Representative images of moderate expression of MAP3K1 in tumor specimens of breast IDC (right upper inset 1000×). c Representative images of high expression of MAP3K1 in tumor specimens of breast IDC (right upper inset, 1000×). d Negative expression of p-ERK in tumor specimens of breast IDC. e Representative images

of moderate expression of p-ERK in tumor specimens of breast IDC (left bottom inset 1000×) f Representative images of high expression of p-ERK in tumor specimens of breast IDC (right upper inset, 1000×). g The scatter plot showed the significant relation between p-ERK expression and MAP3K1 expression (Spearman's correlation, Correlation Coefficient = 0.538; p < 0.001). MAP3K1, mitogen-activated protein kinase kinase kinase 1; p-ERK, phospho-extracellular signal-regulated kinase; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; IDC, infiltrating ductal carcinoma.

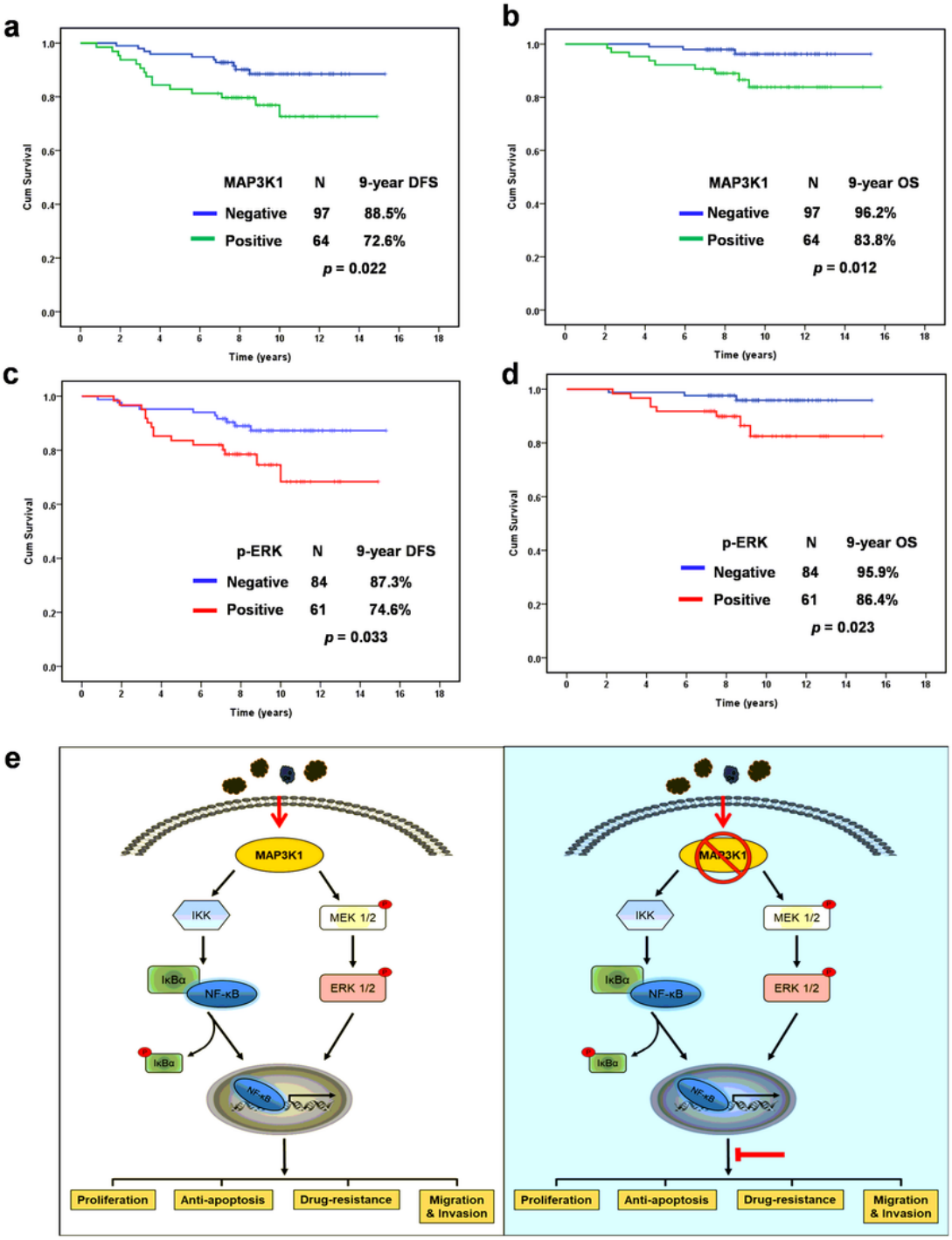

Figure 5

Figure 5

Association between MAP3K1 expression or p-ERK expression in tumor cells and survival of early-stage HR-positive, HER2-negative breast cancer patients. a The DFS for all patients associated with the expression of MAP3K1. b The OS for all patients associated with the expression of MAP3K1. c The DFS for all patients associated with the expression of p-ERK. d The OS for all patients associated with the expression of p-ERK. e MAP3K1 promotes HR-positive, HER2-negative breast cancer cell proliferation, migration, invasion, and resistance to the drugs (docetaxel, doxorubicin, and tamoxifen) through activating ERK and NF-κB 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 and NF-κB signaling. MAP3K1, mitogen-activated protein kinase kinase kinase 1; p-ERK, phospho-extracellular signal-regulated kinase; HR, hormone receptor; HER-2, human epidermal growth factor receptor 2; DFS, disease-free survival; OS, overall survival.

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