EGFR inhibition reverses epithelial-mesenchymal transition, and decreases tamoxifen resistance via Snail and Twist downregulation in breast cancer cells

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Abstract. Tamoxifen resistance remains a major obstacle in the treatment of estrogen receptor (ER)-positive breast cancer. In recent years, the crucial role of the epithelial-mesenchymal transition (EMT) process in the development of drug resistance in breast cancer has been underlined. However, the central molecules inducing the EMT process during the development of tamoxifen resistance remain to be elucidated. In the present study, it was demonstrated that tamoxifen-resistant breast cancer cells underwent EMT and exhibited an enhanced cell motility and invasive behavior. The inhibition of snail family transcriptional repressor 1 (*Snail*) and twist family BHLH transcription factor 1 (*Twist*) reversed the EMT phenotype and decreased the tamoxifen resistance, migration and invasion of tamoxifen-resistant breast cancer cells. In addition, it was observed that the inhibition of epidermal growth factor receptor (EGFR) reversed the EMT phenotype in tamoxifen-resistant MCF7 (MCF-7/TR) cells via the downregulation of *Snail* and *Twist*. Notably, the EGFR inhibitor, gefitinib, decreased tamoxifen resistance, migration and invasion through the inhibition of *Snail* and *Twist*. On the whole, the results of the present study suggest that EGFR may be a promising therapeutic target for tamoxifen-resistant breast cancer. Moreover, it was suggested that gefitinib may serve as a potent novel therapeutic strategy for breast cancer patients, who have developed tamoxifen resistance.

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Abbreviations: ER, estrogen receptor; EMT, epithelial-mesenchymal transition; EGFR, epidermal growth factor receptor; Snai1, snail family transcriptional repressor 1; Twist, twist family BHLH transcription factor 1; Slug, snail family transcriptional repressor 2; MCF-7/TR cells, tamoxifen-resistant MCF-7 cells

Key words: tamoxifen resistance, ER-positive breast cancer, epithelial-mesenchymal transition, Snai1, Twist, epidermal growth factor receptor, gefitinib

Introduction

Breast cancer is the most frequently diagnosed neoplasm and the second leading cause of cancer-related mortality among women worldwide (1,2); four molecular features are used for breast cancer subtypes based on the expression of estrogen receptor (ER), progesterone receptor, human epidermal growth factor receptor (HER)2 and Ki-67 (3). ER-positive breast cancer is the most common clinical subtype, constituting almost 70% of all breast cancer cases (4). Endocrine therapy to block ER activity is the mainstay therapy for ER-positive breast cancer (3,5). Tamoxifen is the most commonly used endocrine treatment for ER-positive breast cancer, particularly for pre-menopausal patients (6). It decreases estrogen-responsive gene transcription by competitively inhibiting the binding of estrogen to ER, thereby suppressing the proliferation of ER-positive breast cancer (7). Treatment with tamoxifen reportedly decreases the risk of recurrence at 5 years by 47% and mortality at 15 years by 34% in patients with early ER-positive breast cancer and prolongs the survival of patients with metastatic breast cancer for ~8 months (8,9). However, ~40% of patients with ER-positive breast cancer develop tamoxifen resistance, leading to metastasis, recurrence and even mortality (10-12). Therefore, tamoxifen resistance plays a main role in the mortality rate of patients with ER-positive breast cancer. Various mechanisms have been proposed to combat this resistance; for example, the modification or loss of ER expression, the upregulation of oncogenic signaling pathways and epigenetic alterations (11,13,14). Nevertheless, the crucial question regarding the definition of therapeutic targets to overcome tamoxifen resistance in ER-positive breast cancer remains unanswered. Therefore, it is important to identify therapeutic targets for overcoming or reversing tamoxifen resistance in ER-positive breast cancer.

In recent years, the importance of the epithelial-mesenchymal transition (EMT) process in the gain of aggressive characteristics in cancers has been recognized (15-17). EMT is a complex process characterized by epithelial cells that lose

cell-cell junctions and acquire mesenchymal properties (18). It is characterized by the downregulated expression of epithelial markers, including E-cadherin, and the upregulated expression of mesenchymal markers, including N-cadherin and vimentin, and EMT-inducing transcription factors, including snail family transcriptional repressor 1 (Snai1), twist family BHLH transcription factor 1 (Twist) and snail family transcriptional repressor 2 (Slug) (19). Several studies have demonstrated that EMT is associated with the gain of migratory and invasive properties, and an increased tolerance to chemotherapy, being also a prominent hallmark of cancer progression (20,21). In addition, the decreased expression of E-cadherin, and the increased expression of N-cadherin and vimentin have been associated with a poor survival in breast, melanoma and prostate cancer (22-24). Furthermore, the EMT phenotype has been identified in a number of cancer cells, including erlotinib-resistant lung cancer cells, doxorubicin-resistant gastric cancer cells and tamoxifen-resistant breast cancer cells (25-27). Therefore, therapeutic strategies based on reversing EMT may provide a novel approach with which to overcome acquired tamoxifen resistance in ER-positive breast cancer.

Tamoxifen-resistant breast cancer cells have been reported to exhibit an EMT phenotype and an EMT gene expression pattern (28,29). Transcription factors, including Snail, Slug and Twist have been reported to mediate EMT by regulating the expression of E-cadherin, N-cadherin and vimentin (30,31). In addition, the dysregulation of EMT-inducing transcription factors exhibits clinical relevance in patients with tamoxifen-resistant breast cancer (32,33). Several growth factor receptors, including fibroblast growth factor 1 receptor (FGFR1), insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), which are involved in the EMT process, are also highly expressed in ER-positive breast cancer cells, supporting the link between EMT and insensitivity to endocrine therapy (34,35). However, the central molecules inducing the EMT process during the development of tamoxifen resistance remain largely unknown.

In the present study, a tamoxifen-resistant MCF-7 (MCF-7/TR) breast cancer cell line was established. MCF-7/TR cells underwent EMT and exhibited an enhanced cell motility and invasive behavior. In addition, *Snail* and *Twist* silencing reversed the EMT phenotype and decreased the tamoxifen resistance, migration and invasion of MCF-7/TR cells. Of note, gefitinib, a known inhibitor of EGFR, reversed EMT and decreased the tamoxifen resistance, migration and invasion of MCF-7/TR cells via the downregulation of *Snail* and *Twist*. The findings of the present study indicate that EGFR may be a promising therapeutic target for tamoxifen-resistant breast cancer treatment. Moreover, it is suggested that gefitinib may serve as a potent novel therapeutic strategy for breast cancer patients, who have developed tamoxifen resistance.

Materials and methods

Reagents. Tamoxifen (MilliporeSigma) and gefitinib (Funakoshi Co., Ltd.) were first dissolved in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corporation) up to a concentration of 50 mM (stock solution) and stored at -20˚C. Stealth small interfering RNA (siRNA) targeting Snail (HSS143995; 5'-CCTCGCTGCCAATGCTCATCTGGGA-3') and Twist (HSS144372; 5'-TGGCGGCCAGGTACATCGACT TCCT-3') were purchased from Thermo Fisher Scientific, Inc. Antibodies against phosphorylated (p)-EGFR (cat. no. 2235; dilution 1:1,000) and EGFR (cat. no. 4267; dilution 1:1,000) were obtained from Cell Signaling Technology, Inc. Antibodies against β-actin (cat. no. A2228; dilution 1:3,000) were purchased from MilliporeSigma. Anti-rabbit secondary antibody (cat. no. 7074; dilution 1:5,000) and anti-mouse secondary antibody (cat. no. 7076; dilution 1:5,000) were obtained from Cell Signaling Technology, Inc.

Cells and cell culture. The tamoxifen-sensitive human breast cancer cell line, MCF-7 (cat. no. JCRB0134), was obtained from the Health Science Research Resources Bank. The MCF-7/TR cell line was established from the MCF-7 cells, following continuous exposure to tamoxifen along with a gradual increase in the concentration from 1 to 25 μM over a period of 6 months. The MCF-7/TR cells were maintained in 25 μM tamoxifen. These cells were cultured in RPMI-1640 (MilliporeSigma) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (FUJIFILM Wako Pure Chemical Corporation), 25 mM HEPES (FUJIFILM Wako Pure Chemical Corporation), 100 μg/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a CO_2 incubator (Sanyo Co., Ltd.) with 95% air and 5% CO_2.

Cell viability assay. Cell viability assay was performed using trypan blue staining. The MCF-7 and MCF-7/TR cells were plated in 96-well plates in RPMI-1640 medium, containing 10% FBS at a concentration of $2x10^3$ cells per well. Subsequently, tamoxifen (0.1, 0.5, 1.5, 10, 25, 50, 100, 250 and 500 μM), Snail siRNA (10 nM), Twist siRNA (10 nM), or gefitinib (1, 5, 10, and 25 μM) were added to the wells. All cells were stained with 0.4% trypan blue (FUJIFILM Wako Pure Chemical Corporation) for 3 min at room temperature, and counted at a magnification of x100 under a light microscope (Olympus CK2; Olympus Corporation) at 3 days. IC50 values were calculated using GraphPad Prism 9.0 (GraphPad Prism software, Inc.).

Transwell invasion and migration assays. For Transwell invasion assay, the Cell Culture Inserts (8.0 μm pore size; Becton, Dickinson and Company) were coated with 20 μl Matrigel (Corning, Inc.) for 30 min at 37˚C. Subsequently, MCF-7 ($5x10^4$ cells) and MCF-7/TR ($5x10^4$ cells) cells previously transfected (as described below) with Snail siRNA (10 nM), Twist siRNA (10 nM), or gefitinib (5 μM) were plated in the upper chamber, and the lower chamber was supplemented with medium containing 10% FBS (Gibco; Thermo Fischer Scientific, Inc.). Following a 24-h incubation, all cells on the upper chamber surface were removed using a wet cotton swab, and those attached on the lower side of the membrane were fixed with 95% ethanol for 10 min at room temperature and stained hematoxylin (MilliporeSigma) for 5 min at room temperature. The cells passing through the Cell Culture Insert were counted at a magnification of x200 under a light microscope (Olympus BX50; Olympus Corporation) in five randomly selected fields. Transwell migration assay was

performed similarly to the Transwell invasion assay, without using Matrigel.

Reverse transcription-quantitative PCR (RT-qPCR). The MCF-7/TR cells were cultured with Snail siRNA (10 nM), Twist siRNA (10 nM), or gefitinib (5 μM). Total RNA extraction from the MCF-7 and MCF-7/TR cells was performed using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription reactions were performed using the PrimeScript RT reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol under the following thermocycling conditions: 37˚C for 15 min, followed by 85˚C for 5 sec. qPCR was performed using TB Green Premix Ex Taq (Takara Bio, Inc.) and an ABI Prism 7000 detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers for RT-qPCR were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The following primer sequences were used: E-cadherin forward, 5'-GAACGCATTGCCACATACAC-3' and reverse, 5'-GAATTCGGGCTTGTTGTCAT-3'; N-cadherin forward, 5'-CTCCTATGAGTGGAACAGGAACG-3' and reverse, 5'-TTGGATCAATGTCATATTCAAGTGCTGTA-3'; vimentin forward, 5'-AGATGGCCCTTGACATTGAG-3' and reverse, 5'-CCAGAGGGAGTGAATCCAGA-3'; Snail forward, 5'-GCGAGCTGCAGGACTCTAAT-3' and reverse, 5'-GGACAGAGTCCCAGATGAGC-3'; Slug forward, 5'-CGTTTTTCCAGACCCTGGTT-3' and reverse, 5'-CTGCAGATGAGCCCTCAGA-3'; Twist forward, 5'-CGCCCCGCTCTTCTCCTCT-3' and reverse, 5'-GACTGTCCATTTTCTCCTTCTCTG-3'; GAPDH was used as an internal control and the following primer sequences were used: GAPDH forward, 5'-ACTTTGTCAAGCTCATTT-3' and reverse, 5'-TGCAGCGAACTTTATTG-3'. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Cq}$ method (36).

RNA interference/transfection. The MCF-7/TR cells were transfected with 10 nM Snail siRNA, 10 nM Twist siRNA and 10 nM Stealth™ RNAi Negative Control (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.). Lipofectamine 3000 and siRNAs were diluted in RPMI-1640 medium, respectively, and were incubated for 5 min at room temperature. The diluted Lipofectamine 3000 and siRNAd were then mixed at a ratio of 1:1, and subsequently they were incubated for 15 min at room temperature. Subsequently, the complexes were added to the cells followed by incubation for 48 h at 37˚C in a 5% CO_2. Following transfection, the cells were treated according to the subsequent experimental protocol requirements.

Receptor tyrosine kinase (RTK) analysis. RTK analyses were conducted using the 7-Plex RTK Mitogenesis Phosphoprotein Magnetic Bead kit (cat. no. 48-671MAG; Merck Life Science UK, Ltd.) according the manufacturer's protocol. Briefly, the MCF-7 and MCF-7/TR cells were collected and lysed using lysis buffer [20 mM Tris-HCl pH 8.0 (FUJIFILM Wako Pure Chemical Corporation), 150 mM NaCl (FUJIFILM Wako Pure Chemical Corporation), 2 mM ethylenediaminetetraacetic acid (EDTA; FUJIFILM Wako Pure Chemical Corporation), 100 mM NaF, 1% NP40 (both from FUJIFILM Wako Pure Chemical Corporation), 1 μg/ml leupeptin (MilliporeSigma), 1 μg/ml antipain (MilliporeSigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (MilliporeSigma)]. The samples were mixed with 7-Plex RTK Mitogenesis magnetic beads and incubated overnight at 4˚C. Subsequently, the samples were washed and mixed Biotin-Labeled Detection Antibody (dilution 1:20; cat. no. 48-671MAG; Merck Life Science UK, Ltd.). RTK expression was measured using the Luminex® 200 instrument (Luminex Corporation).

Western blot analysis. The MCF-7 and MCF-7/TR cells were cultured with gefitinib (5 μM). Subsequently, the MCF-7 and MCF-7/TR cells were collected and lysed with lysis buffer [20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 μM pepstatin, 1 μM leupeptin, 2 mM sodium orthovanadate, 1 μM calpain inhibitor, phosphatase inhibitor cocktail I/II and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein samples were quantified using the BCA Protein assay kit (Thermo Fischer Scientific, Inc.). The extracts (40 μg) were separated using 10% sodium dodecyl sulfate (FUJIFILM Wako Pure Chemical Corporation)-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a transfer to polyvinylidene fluoride (PVDF) membranes (Cytiva). The membranes were blocked with 5% skim milk for 30 min at room temperature and incubated with the primary antibodies (as indicated above in the '*Reagents*' paragraph) overnight at 4˚C. The membranes were then incubated with secondary antibodies (as indicated above in the '*Reagents*' paragraph) for 2 h at room temperature. The immunoreactive bands were visualized using Luminata Forte Western HRP substrate (Merck Life Science UK, Ltd.). β-actin was used as the loading control. The bands were analyzed using Densitograph software CS Analyzer ver 3.0 (Atto Corporation).

Statistical analysis. GraphPad Prism 9.0 (GraphPad Prism software, Inc.) was used for analysis. All data are expressed as the mean ± standard deviation (SD). Data comparisons between two groups were performed using an unpaired Student's t-test. Comparisons among multiple groups were performed using analysis of variance (ANOVA) followed by Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MCF-7/TR cells exhibit an enhanced motility and invasive behavior. To confirm whether MCF-7/TR cells acquired a tamoxifen-resistant phenotype, parental MCF-7 and MCF-7/TR cells were treated with various concentrations of tamoxifen for 72 h. Tamoxifen decreased the viability of the MCF-7 cells; however, it exerted a limited effect on the viability of MCF-7/TR cells (Fig. 1A). The IC50 value was 8.0 μM for the parental MCF-7 cells and 107.2 μM for the MCF-7/TR cells. Subsequently, it was examined whether the acquisition of a tamoxifen-resistant phenotype enhances cell motility and invasive behavior. It was observed that the MCF-7/TR cells exhibited a significantly increased migratory and invasive ability in comparison with the MCF-7 cells (Fig. 1B and C). These results indicated that the MCF-7/TR cells exhibit an enhanced motility and invasive behavior.

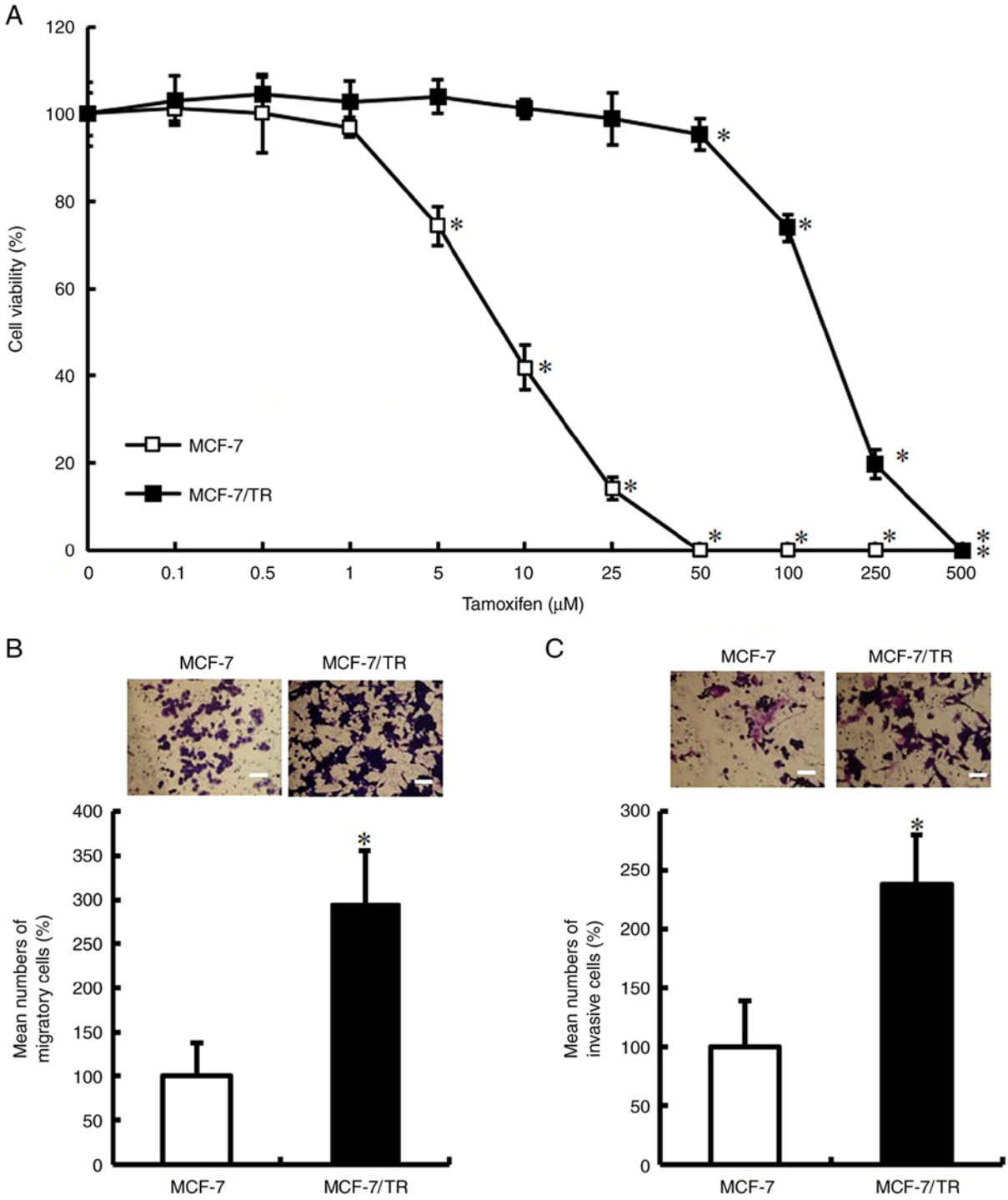

Figure 1. MCF-7/TR cells exhibit an enhanced cell motility and invasive behavior. (A) MCF-7 and MCF-7/TR cells were treated with tamoxifen (0.1-500 μM) for 72 h and subsequently stained with trypan blue. The number of stained cells was counted on day 3. Results are presented as the mean ± SD of three independent experiments. *P<0.05, as compared to the untreated MCF-7 or MCF-7/TR cells. (B and C) Cell migration was analyzed using Transwell culture inserts, whereas cell invasion was analyzed using Transwell culture inserts coated with Matrigel. Results are presented as the mean ± SD of three independent experiments. Representative images of invasion assay of MCF-7 and MCF-7/TR cells are presented on the top panels. Magnification, x20. Scale bar, 50 μm. *P<0.05 as compared to MCF-7 cells. MCF-7/TR, tamoxifen-resistant MCF-7 cells.

MCF-7/TR cells acquire the EMT phenotype. To determine whether the MCF-7/TR cells acquired the EMT phenotype, morphological changes in the MCF-7/TR cells were examined. The MCF-7/TR cells exhibited a spindle shape, intercellular spaces and scattering, whereas the MCF-7 cells exhibited firmly packed cobblestone-like clusters (Fig. S1A). Moreover, *E-cadherin* expression was downregulated, and *N-cadherin* and *vimentin* expression was upregulated in the MCF-7/TR cells, but not in the MCF-7 cells (Fig. 2). These results indicated that the MCF-7/TR cells acquired the EMT phenotype.

Silencing of Snail and Twist reverses the EMT phenotype in MCF-7/TR cells. Snail, Slug and Twist are three

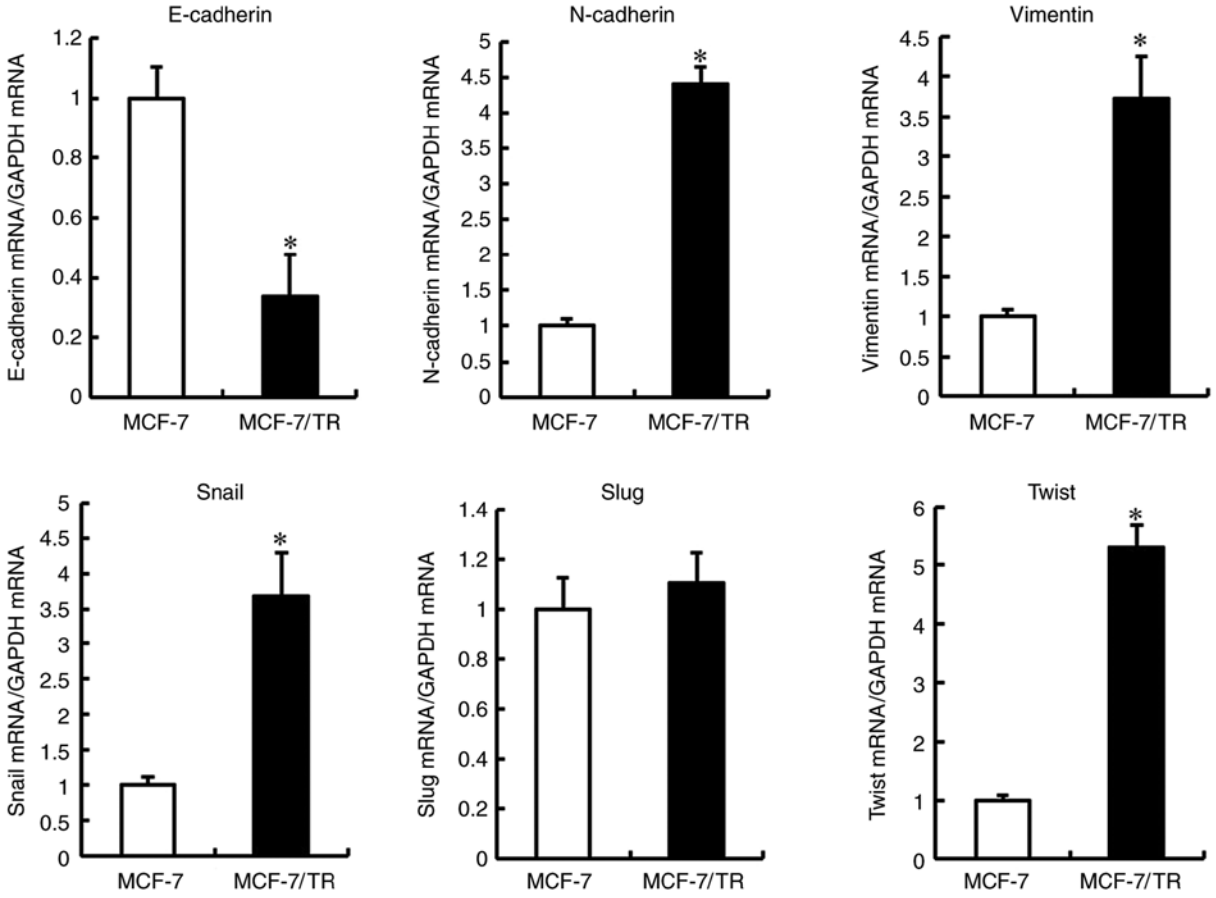

Figure 2. MCF-7/TR cells acquire the epithelial-mesenchymal transition phenotype. *E-cadherin*, *N-cadherin*, *vimentin*, *Snail*, *Slug* and *Twist* mRNA expression was measured using reverse transcription-quantitative PCR. Data are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to MCF-7 cells. Snai1, snail family transcriptional repressor 1; Twist, twist family BHLH transcription factor 1; Slug, snail family transcriptional repressor 2; MCF-7/TR, tamoxifen-resistant MCF-7 cells.

well-documented EMT regulatory transcription factors. Therefore, the present study examined their expression levels in MCF-7 and MCF-7/TR cells. *Snail* and *Twist* expression levels were upregulated in the MCF-7/TR cells compared with the MCF-7 cells, while *Slug* expression was not significantly unaltered (Fig. 2). Furthermore, it was investigated whether Snail and Twist silencing reversed the EMT phenotype in MCF-7/TR cells. Transfection with Snail and Twist siRNA induced morphological changes, resulting in EMT in MCF-7/TR cells (Fig. S1B). In addition, *Snail* and *Twist* silencing resulted in *E-cadherin* upregulation, and *N-cadherin* and *vimentin* downregulation (Fig. 3). These results indicated that the silencing of Snail and Twist may reverse the EMT phenotype in MCF-7/TR cells.

Silencing of Snail and Twist decreases the tamoxifen resistance, migration and invasion of MCF-7/TR cells. The present study then examined whether the inhibition of Snail and Twist impaired tamoxifen resistance, and decreased the migration and invasion of MCF-7/TR cells. It was revealed that transfection with Snail and Twist siRNA impaired the tamoxifen resistance of MCF-7/TR cells (Fig. 4A). In addition, Snail and Twist siRNA inhibited cell migration and invasion (Fig. 4B and C). These results indicated that the silencing of Snail and Twist may decrease tamoxifen resistance, migration, and invasion in MCF-7/TR cells.

Inhibition of EGFR reverses the EMT phenotype in MCF-7/TR cells by downregulating Snail and Twist expression. The molecular mechanisms underlying the increased expression levels of Snail and Twist in the MCF-7/TR cells have not yet been fully elucidated. Recent research has reported that several RTKs, including EGFR, IGF1R and fibroblast growth factor 1 receptor, which are involved in the EMT process, are highly expressed in tamoxifen-resistant breast cancer, supporting the link between EMT and insensitivity to endocrine therapy (37). Therefore, the present study examined RTK expression in MCF-7 and MCF-7/TR cells using Luminex® 200. It was revealed that EGFR expression was higher in the MCF-7/TR cells in comparison with the MCF-7 cells (Fig. 5). However, no changes in the expression of c-Met, IGF1R, insulin receptor (IR), HER3 and HER4 proteins were observed between the MCF-7 and MCF-7/TR cells. It was then examined whether EGFR inhibition reversed the EMT phenotype through Snail and Twist inhibition. Firstly, the effect of the EGFR inhibitor, gefitinib, on the viability of MCF-7 and MCF-7/TR cells was examined using trypan blue exclusion assay. The MCF-7 cells treated with 1, 5 and 10 μM gefitinib, and the MCF-7/TR cells treated with 1 and 5 μM gefitinib did not exhibited an inhibition of cell viability (Fig. 6A). However, the MCF-7 cells treated with 25 μM gefitinib, and the MCF-7/TR cells treated with 10 and 25 μM gefitinib exhibited a decrease in cell viability

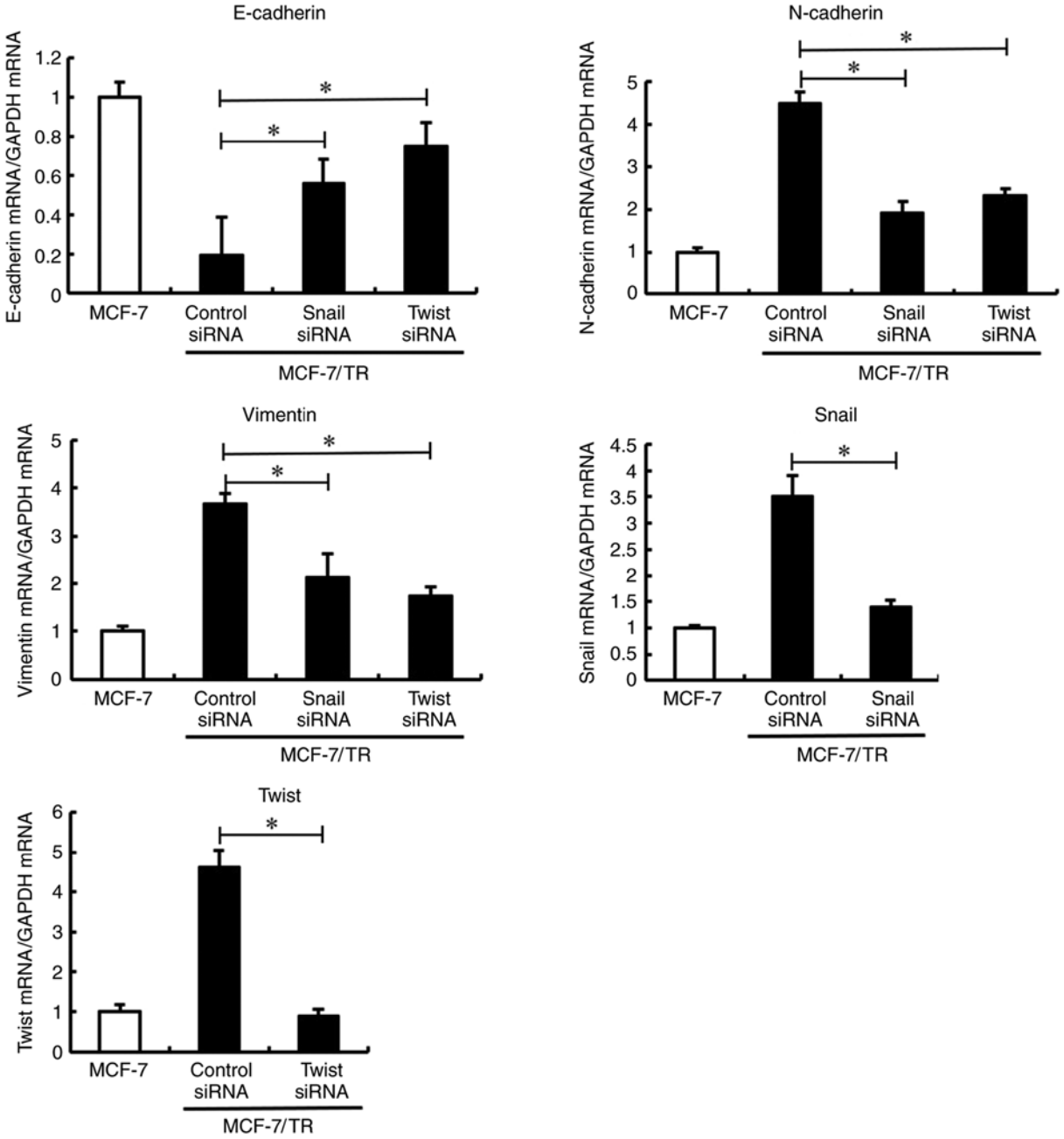

Figure 3. Snail and Twist inhibition reverses the epithelial-mesenchymal transition phenotype in MCF-7/TR cells. MCF-7/TR cells were transfected with Snail siRNA (10 nM), Twist siRNA (10 nM), or Stealth™ RNAi Negative Control (siRNA control) for 3 days. *E-cadherin*, *N-cadherin*, *vimentin*, *Snail*, and *Twist* mRNA expression levels were measured using reverse transcription-quantitative PCR. Data are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to the siRNA control. The F values are 43.6 (E-cadherin), 79.52 (N-cadherin), 41.13 (Vimentin), 46.48 (Snail) and 158.5 (Twist). Snai1, snail family transcriptional repressor 1; Twist, twist family BHLH transcription factor 1; Slug, snail family transcriptional repressor 2; MCF-7/TR, tamoxifen-resistant MCF-7 cells.

compared to the untreated cells. In addition, the expression of EGFR in the gefitinib-treated MCF-7 and MCF-7/TR cells was examined using western blot analysis. It was revealed that gefitinib suppressed the expression of p-EGFR (Figs. 6B and S2). These results revealed that 5 μM gefitinib did not inhibit cell viability, whereas at a concentration >10 μM, it inhibited the viability of the MCF-7/TR cells. Therefore, the MCF-7/TR cells were treated with gefitinib at 5 μM in subsequent experiments. It was thus demonstrated that gefitinib may reverse the EMT phenotype through the inhibition of *Snail* and *Twist* (Figs. 7 and S1A). These results suggested that EGFR inhibition reversed the EMT phenotype in MCF-7/TR cells via the downregulation of Snail and Twist.

Inhibition of EGFR decreases the tamoxifen resistance, migration and invasion of MCF-7/TR cells. The present study then examined whether gefitinib decreases the tamoxifen resistance, migration and invasion of MCF-7/TR cells. Gefitinib treatment was found to decrease the tamoxifen resistance of MCF-7/TR cells (Fig. 8A). The combination of tamoxifen and gefitinib slightly reduced the viability of the MCF-7 cells compared to the tamoxifen-treated MCF-7 cells.

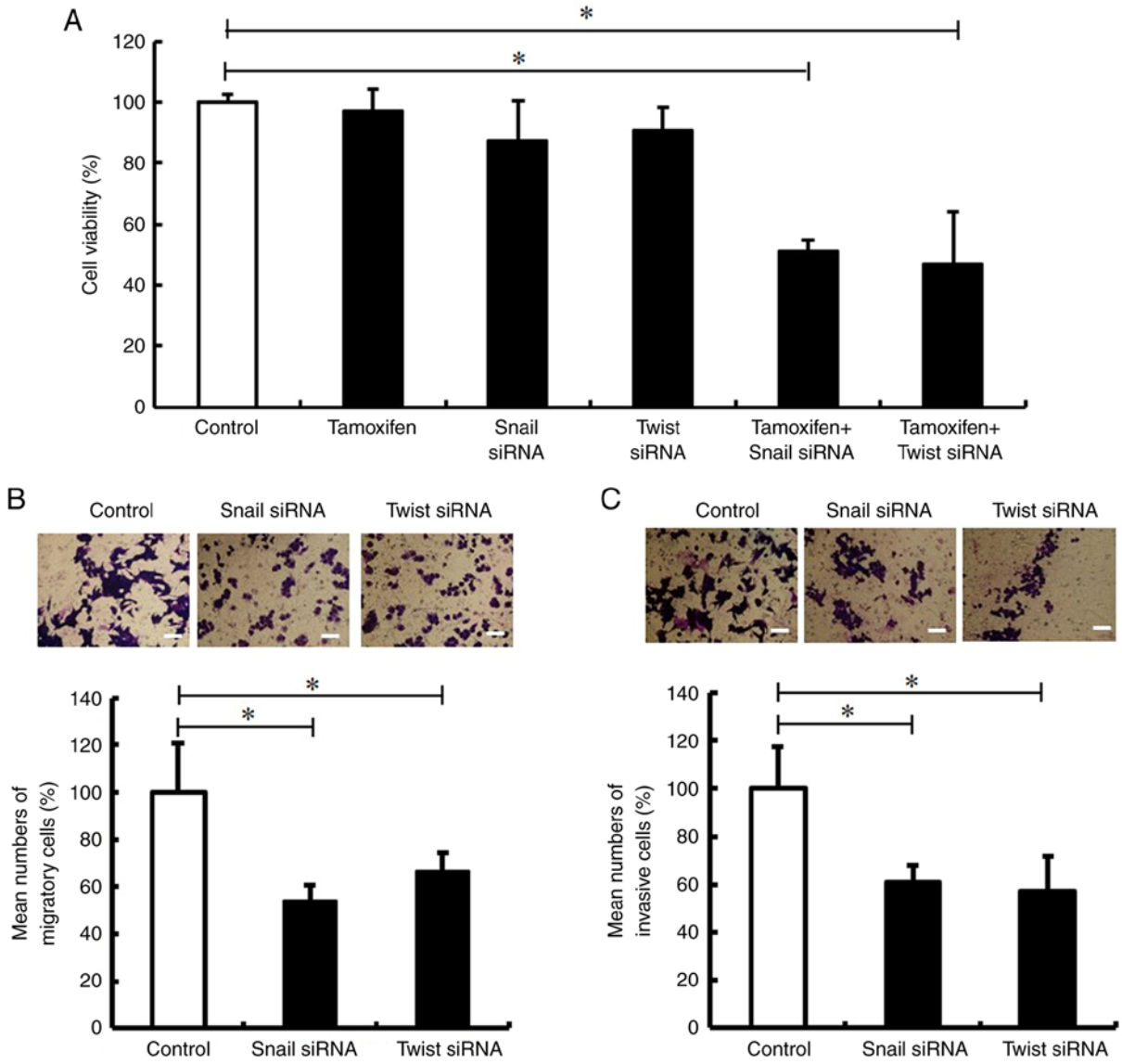

Figure 4. Snail and Twist inhibition decreases the tamoxifen resistance, migration and invasion of MCF-7/TR cells. MCF-7/TR cells were transfected with Snail siRNA (10 nM), Twist siRNA (10 nM), or Stealth™ RNAi Negative Control (Control) for 3 days. (A) MCF-7/TR cells were treated with 25 μM tamoxifen. The cells were stained with trypan blue, and the number of stained cells was counted on day 3. The results are presented as the mean ± SD of three independent experiments. *P<0.05 compared to the control. The F value is 25.29. (B and C) Cell migration was analyzed using Transwell culture inserts, whereas cell invasion was analyzed using Transwell culture inserts coated with Matrigel. Representative images of invasion assay of MCF-7/TR cells are presented on the top panels. Magnification, x20. Scale bar, 50 μm. The results are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to the control. The F value is 27.22 (cell migration) and 12.32 (cell invasion). Snai1, snail family transcriptional repressor 1; Twist, twist family BHLH transcription factor 1; MCF-7/TR, tamoxifen-resistant MCF-7 cells.

In addition, Transwell invasion and migration assays revealed that gefitinib treatment inhibited the migration and invasion of MCF-7/TR cells (Fig. 8B and C). However, no changes were observed in the migration and invasion of the gefitinib-treated MCF-7 cells. These results indicated that gefitinib may successfully decrease the tamoxifen resistance, migration and invasion of MCF-7/TR cells.

Discussion

Tamoxifen has been used in the treatment of both pre- and post-menopausal patients with ER-positive breast cancer for >40 years. However, ~40% of ER-positive breast cancer patients develop resistance to tamoxifen (10). Numerous studies have been conducted to identify the underlying mechanisms of tamoxifen resistance in various research and clinical settings (11,14,38,39). EMT has been reported to contribute to drug resistance, an increased motility and cancer metastasis in a variety of cancer types, including breast, pancreatic, and colorectal cancers (40). Furthermore, tamoxifen-resistant breast cancer cells undergo EMT morphological changes, which alters their growth rate and increases aggressive behavior (41,42). Additionally, restoring E-cadherin expression or reversing EMT in resistant cancer cells has been reported to enhance cancer cell susceptibility to chemotherapy and radiotherapy (43). Taken together, therapeutic strategies that reverse EMT may be a novel approach which may be used to overcome acquired tamoxifen resistance in breast cancer.

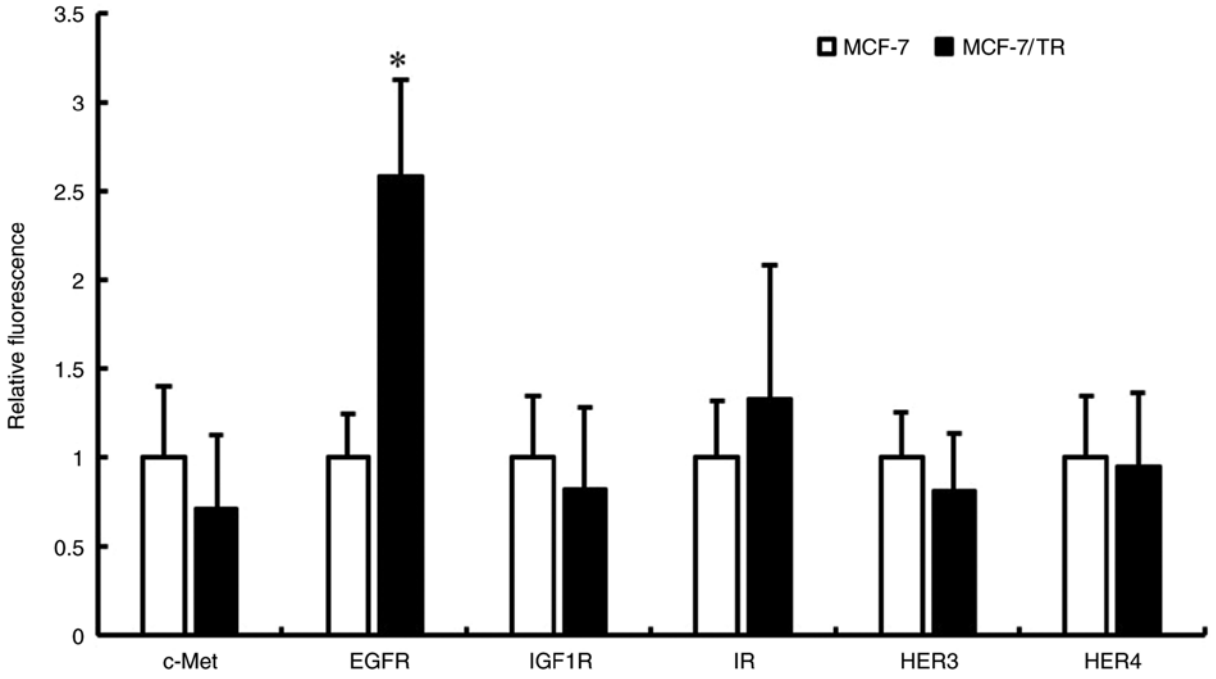

Figure 5. MCF-7/TR cells exhibit an increased EGFR phosphorylation in comparison with MCF-7 cells. c-Met, EGFR, IGF-1R, IR, HER3 and HER4 protein phosphorylation levels were measured using Luminex® 200. Data are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to MCF-7 cells. MCF-7/TR, tamoxifen-resistant MCF-7 cells; c-Met, tyrosine-protein kinase Met; HER, human epidermal growth factor receptor; EGFR, epidermal growth factor receptor; IGF1R, insulin-like growth factor 1 receptor; IR, insulin receptor.

However, crucial questions concerning the central molecules controlling the EMT process during the development of tamoxifen resistance remain unanswered.

In the present study, a tamoxifen-resistant breast cancer cell line, MCF-7/TR, was established, that exhibited an enhanced cell motility and invasive behavior. In addition, an increased expression of the mesenchymal protein, vimentin, and a decreased expression of the epithelial marker, E-cadherin, were revealed, as well as morphological changes consistent with EMT. It was also demonstrated that Snail and Twist silencing may reverse the EMT phenotype, and decrease the tamoxifen resistance, migration and invasion of MCF-7/TR cells. Increased Snail expression levels may induce an EMT phenotype, and increased migration and invasion in various physiological and pathological settings (44-46). The expression of Twist has also been found to be associated with various aggressive cancer types, including breast, gastric and bladder cancer (47-51). Previous studies have reported that Snail and Twist may function by inducing epigenetic silencing at the E-cadherin promoter in the form of hypermethylation and histone deacetylation (40,44,45-54). Twist overexpression has been reported to increase the expression of protease-activated receptor 1 (PAR1), and promote the EMT, migration and invasion of ER-positive breast cancer cells (55). The results of the present study suggested that Snail and Twist may be important targets for overcoming tamoxifen resistance, and controlling cancer migration and invasion.

Tamoxifen-resistant breast cancer is unresponsive to the majority of targeted clinical therapies; thus, there is an urgent need for alternative therapies. Therapeutic strategies based on the reversal of EMT may be a novel approach for overcoming acquired tamoxifen resistance in breast cancer. The RTK signaling pathway has been demonstrated to contribute to EMT and tumor cell invasion (56). The activation of RTK and its downstream signaling effectors, including MAPK or PI3K, is crucial for an increased rate of cell proliferation in epithelial cells (57). In the present study, it was demonstrated that EGFR expression was increased in MCF-7/TR cells in comparison with MCF-7 cells. Notably, the EGFR inhibitor, gefitinib, reversed the EMT phenotype through the inhibition of Snail and Twist. In addition, gefitinib decreased the tamoxifen resistance, migration and invasion of MCF-7/TR cells. EGFR is an important transmembrane protein that is involved in normal epithelial development, as well as in tumor cell proliferation, migration and metastasis. It has been reported to be overexpressed in breast cancer, particularly in more aggressive breast tumor phenotypes associated with poor disease prognosis (58-60). Furthermore, EGFR activation has been reported to induce EMT in cancer cells via the upregulation of Snail and Twist (61,62). The findings of the present study indicated that EGFR activation was an independent biomarker in tamoxifen-resistant breast cancer, and a potential novel therapeutic target that may contribute to reversing EMT and re-sensitizing breast cancer cells to tamoxifen treatment.

Increased knowledge of the signaling factors and pathways inducing tamoxifen resistance could not only aid in the discovery of novel drug targets in ER-positive breast cancer, but also in expanding further the use of presently available medications. Although a number of studies have revealed that tamoxifen resistance promotes EMT-like behavior, the underlying molecular mechanisms and the participating cellular signaling pathways have not yet

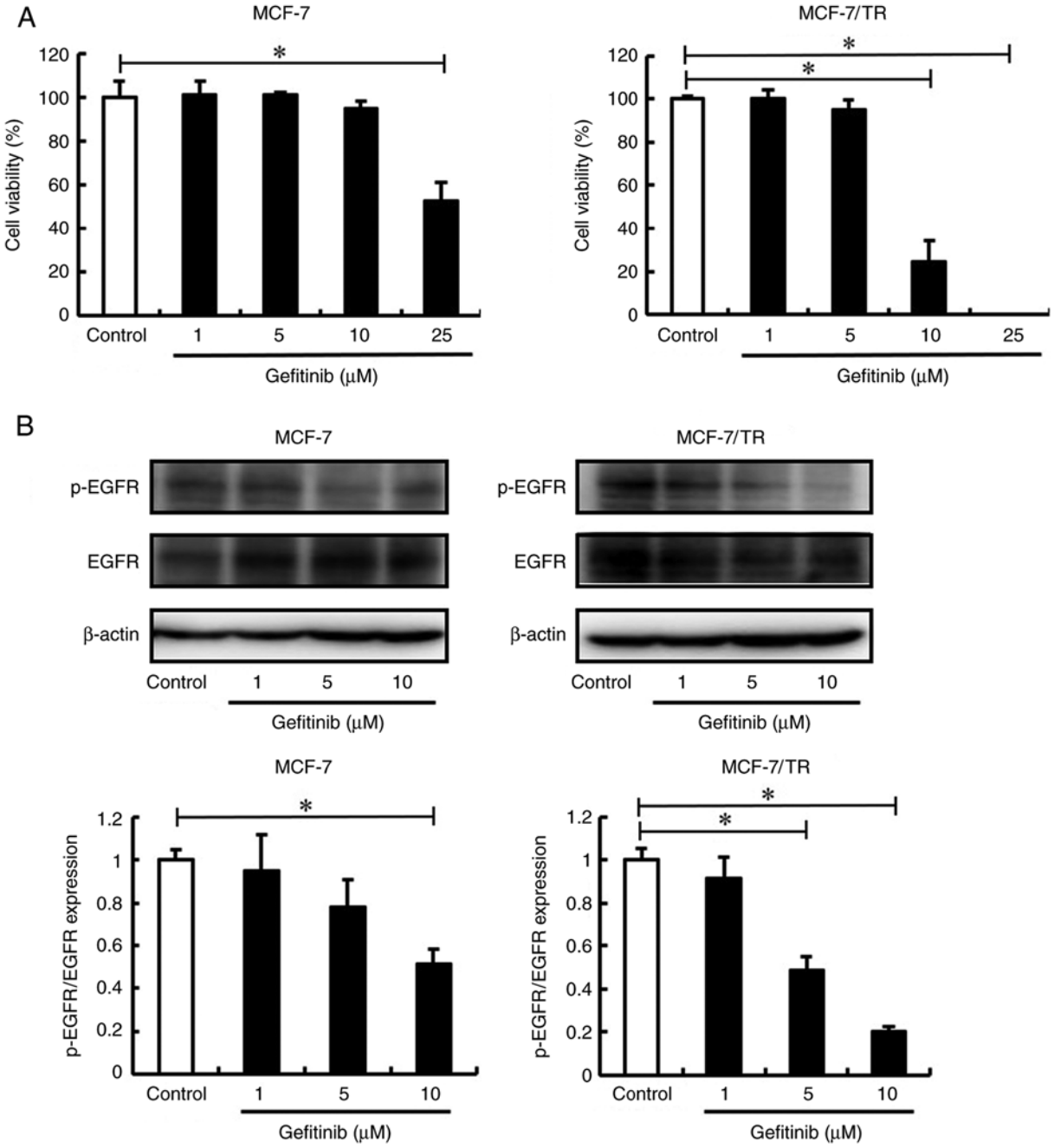

Figure 6. EGFR inhibition decreases MCF-7/TR cell viability. (A) MCF-7 and MCF-7/TR cells were treated with gefitinib (1, 5, 10 and 25 μM), stained with trypan blue and the number of stained cells was counted on day 3. The results are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to the untreated control. The F values are 151 (MCF-7) and 231.5 (MCF-7/TR). (B) MCF-7 and MCF-7/TR cells were treated with gefitinib (1, 5 and 10 μM) for 2 days. The expression of phosphorylated EGFR and EGFR was evaluated by using western blot analysis. β-actin was used as an internal control. Bands were normalized to EGFR. Data are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to the untreated control. The F values are 13.49 (MCF-7) and 445.3 (MCF-7/TR). EGFR, epidermal growth factor receptor; MCF-7/TR, tamoxifen-resistant MCF-7 cells.

been studied in detail (63-65). In the present study, it was indicated that EGFR is a promising therapeutic target for tamoxifen-resistant breast cancer. In addition, the EGFR inhibitor, gefitinib, decreased tamoxifen resistance, migration and invasion through the inhibition of *Snail* and *Twist*. Gefitinib has been approved by the FDA for the treatment of metastatic non-small cell lung cancer. Moreover, gefitinib is well-tolerated and has been shown to be effective in treating acquired tamoxifen-resistance in breast cancer patients in a phase II study (66). Therefore, gefitinib may serve as a potent novel therapeutic strategy for breast cancer patients, who have developed tamoxifen resistance. In addition, repurposing gefitinib may be a more effective and inexpensive approach than traditional drug development.

The present study has a few limitations, however. The present study clarified that the EGFR inhibitor, gefitinib, reversed the EMT phenotype through the inhibition of Snail and Twist. Consistent with these findings, Hiscox *et al* (41) reported that the inhibition of EGFR may alter the EMT-like phenotype in tamoxifen-resistant breast cancer

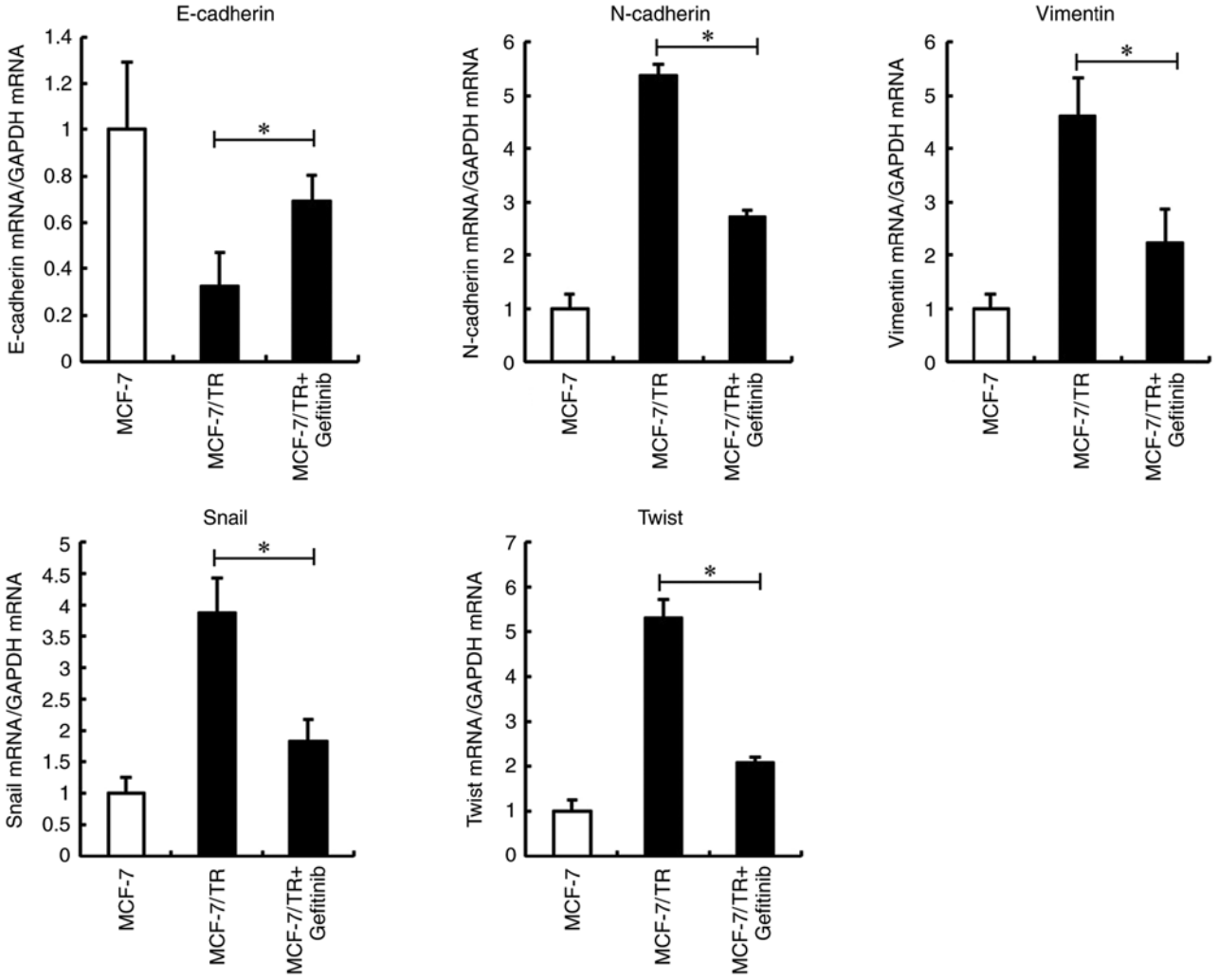

Figure 7. EGFR inhibition reverses the EMT phenotype in MCF-7/TR cells via Snail and Twist downregulation. MCF-7/TR cells were treated with gefitinib (5 μM) for 3 days. *E-cadherin*, *N-cadherin*, *vimentin*, *Snail*, and *Twist* levels were measured using reverse transcription-quantitative PCR. Data are presented as the mean ± SD of three independent experiments. *P<0.05 as compared to MCF-7/TR cells. The F values are 236.4 (E-cadherin), 483.8 (N-cadherin), 49.86 (Vimentin), 49.87 (Snail), and 220.3 (Twist). EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; MCF-7/TR, tamoxifen-resistant MCF-7 cells; Snai1, snail family transcriptional repressor 1; Twist, twist family BHLH transcription factor 1; Slug, snail family transcriptional repressor 2.

cells. However, previous studies concerning the association between EGFR and EMT in tamoxifen resistance have been contradictory. Jiang *et al* (67) reported that the inhibition of the EGFR pathway, which successfully restored the tamoxifen sensitivity of Snail-expressing breast cancer cells, could not reverse their mesenchymal phenotype. In the present study, tamoxifen-resistant MCF-7 cells were used, established from the MCF-7 cells, following a continuous exposure to tamoxifen and a gradual increase in the tamoxifen concentration. By contrast, Jiang *et al* (67) used stable Snail-overexpressing breast cancer cells (MCF-7 and T47D). Therefore, these inconsistent results may be attributed to the methods of tamoxifen-resistant breast cancer cell establishment. The association between EGFR and EMT warrants further investigations using tamoxifen-resistant breast cancer cell studies. In addition, the EGFR inhibition efficacy in MCF/TR cells should be validated *in vivo*.

In conclusion, the present study demonstrated that tamoxifen-resistant breast cancer cells may undergo EMT, and exhibit an enhanced cell motility and invasive behavior. Snail and Twist silencing reversed the EMT phenotype, and decreased tamoxifen resistance, migration and invasion. More importantly, the EGFR inhibitor, gefitinib, may be capable of reversing the EMT phenotype through the inhibition of Snail and Twist, and enhancing tamoxifen susceptibility in breast cancer cells. Taken together, the results of the present study suggest that EGFR may be a promising therapeutic target in tamoxifen-resistant breast cancer, and gefitinib may have potential clinical treatment applications.

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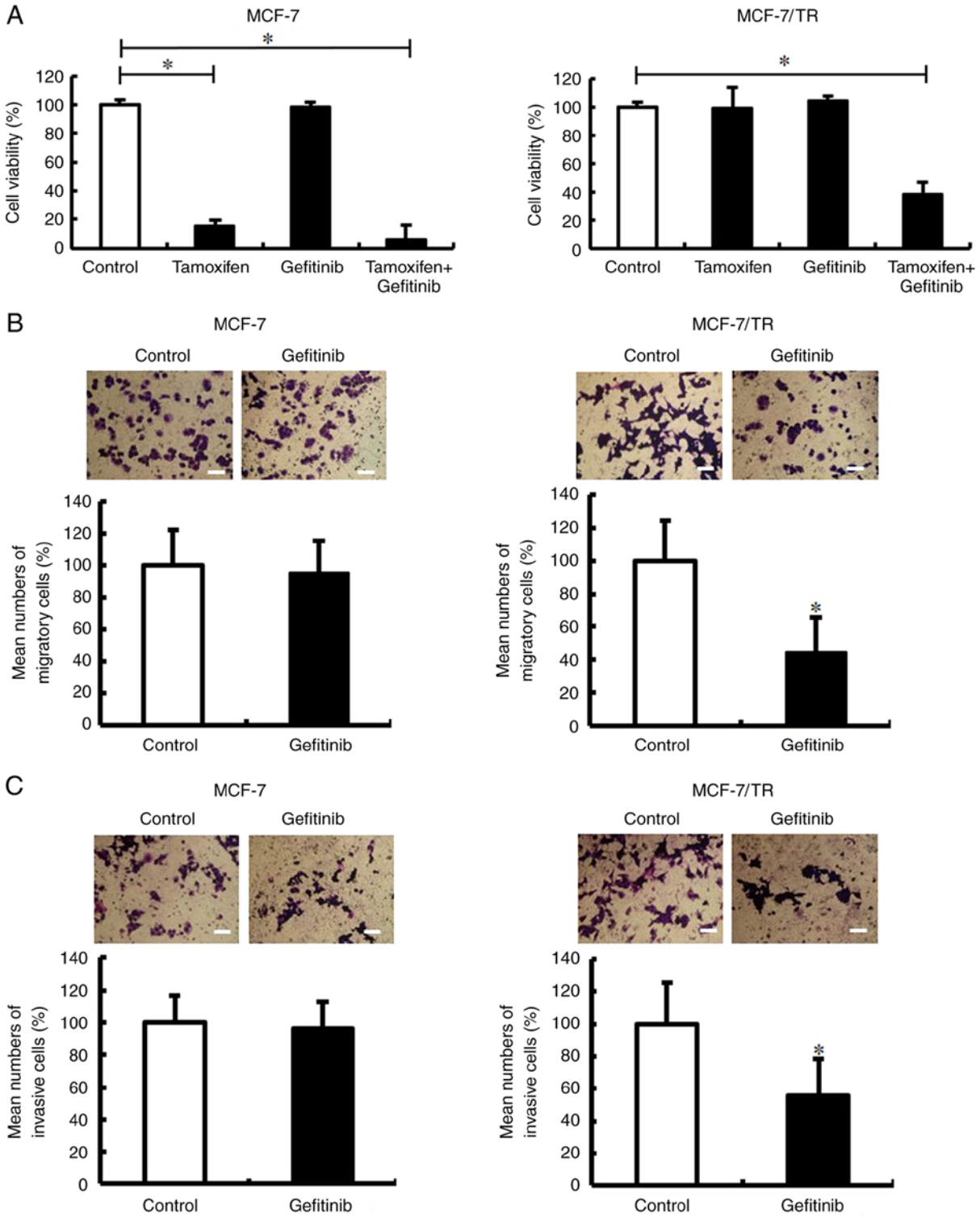

Figure 8. EGFR inhibition decreases the tamoxifen resistance, migration and invasion of MCF-7/TR cells. MCF-7 and MCF-7/TR cells were treated with gefitinib (5 μM) for 3 days. (A) MCF-7 and MCF-7/TR cells were treated with 25 μM tamoxifen. These cells were stained with trypan blue and the number of stained cells was counted on day 3. The results are presented as the mean ± SD of three independent experiments. *P<0.05 compared to the untreated control. The F values are 218.5 (MCF-7) and 30.71 (MCF-7/TR). (B and C) Cell migration was analyzed using Transwell culture inserts, whereas cell invasion was analyzed using Transwell culture inserts coated with Matrigel. Representative images of invasion assay of MCF-7 and MCF-7/TR cells are presented on the top panels. Magnification, x20. Scale bar, 50 μm. The results are presented as means ± SD of three independent experiments. *P<0.05 as compared to the untreated control. EGFR, epidermal growth factor receptor; MCF-7/TR, tamoxifen-resistant MCF-7 cells.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TT wrote the manuscript, and performed the cell viability assay, Transwell and migration assays, RNA interference/transfection

assays, western blot analysis and the statistical analyses. MTs performed the cell viability assay, RT-qPCR and RNA interference/transfection assays. TM, AK, MJ and TO performed the Transwell and migration assays, RT-qPCR and western blot analysis. MTa contributed to the statistical analyses. SN conceptualized and coordinated the present study. All authors have read and approved the final manuscript. SN and TT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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