

Rapamycin enhances the sensitivity of ER-positive breast cancer cells to tamoxifen by upregulating p73 expression

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Abstract. A total of 70% of breast cancers express the estrogen receptor (ER) α ; therefore, targeting the ER may be an effective endocrine therapy with which to inhibit breast cancer growth. Tamoxifen is the most common-used clinically used drug for the treatment of advanced or metastatic ER-positive (ER⁺) breast cancer. However, a substantial proportion of patients become resistant to endocrine therapies. To overcome this limitation, in this study, we sought to maximize the benefits associated with tamoxifen therapy via drug combination strategies. We demonstrated that rapamycin, an FDA-approved mammalian target of rapamycin (mTOR) inhibitor, enhanced the effects of endocrine therapy with tamoxifen, and the concentration of tamoxifen required for ER⁺ breast cancer cell growth inhibition was substantially reduced. Moreover, treatment with rapamycin plus tamoxifen significantly inhibited tumor growth *in vivo*. In addition, this synergistic effect may be mediated by the induction of p73. We revealed a novel mechanism in which p73 increases ER α expression by directly binding to the promoter region of the ER α gene. Taken together, the findings of this study indicate that combination therapy with rapamycin and tamoxifen underlying p73-mediated ER α expression may provide new insight into the drug combination for the treatment of ER⁺ breast cancer.

Introduction

Breast cancer is a complex disease and the major cause of cancer-related mortality among women. Approximately 70% of breast cancers express the estrogen receptor (ER) and are thus termed ER-positive (ER⁺) breast cancers, which represent the primary luminal molecular subtype of breast cancer (1-3). Generally, patients with ER⁺ breast cancer have a better efficacy and prognosis than those with ER-negative (ER⁻) breast cancer (4). ER can be categorized into two structurally related genes, ER α and ER β , in which ER α is a major regulator of breast cancer development and progression (5,6). Therefore, therapies targeting ER, known as endocrine therapies, have become the mainstay of prevention and treatment of all stages of ER⁺ breast cancers (7,8). The common drugs used in endocrine therapy in breast cancer include the following: i) Selective ER modulators (SERM), such as tamoxifen, which directly inhibit ERs by selecting estrogen modulators with mixed agonistic/antagonistic activities; ii) selective ER downregulators (SERD), such as fulvestrant, which inhibit ER signaling through the degradation of ER expression; iii) aromatase inhibitors, such as letrozole, which deprive the receptor's ligand by blocking estrogen production (9,10).

Tamoxifen, the first-line endocrine drug used in the treatment of ER⁺ breast cancer, has contributed to a marked increase in the long-term survival rate (11,12). Nevertheless, a considerable proportion of patients with localized breast cancers and metastatic breast cancers become resistant to endocrine therapies (13). In view of this, high-dose tamoxifen (>100 mg daily) is used in place of standard-dose tamoxifen (20 mg daily) for the treatment of the above-mentioned breast cancers (14). However, this treatment is associated with severe side-effects, including hyperplasia, venous thromboembolic disease (15) and acquired tamoxifen resistance (16,17).

To overcome this limitation, novel strategies to reduce the dose of tamoxifen, while still maintaining its anticancer functions are currently under investigation (18). Some clinically used drugs, which were not developed for the treatment of cancer previously, may have some antitumor effects which may enhance the sensitivity of ER⁺ tumors to tamoxifen. For example, the anti-diabetic drug, metformin, has been shown to

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enhance the tamoxifen-mediated tumor growth inhibition of ER⁺ breast cancer (19). The selective cyclooxygenase (COX)-2 inhibitor, celecoxib, has also been shown to alleviate the tamoxifen-induced angiogenic effects in metastatic ER⁺ breast cancer (20). Recently, it has been reported that the activation of mammalian target of rapamycin (mTOR) signaling leads to multiple agent therapeutic resistance in ER⁺ breast cancer (21). Rapamycin, an mTOR inhibitor, which is a macrolide immunosuppressant and was originally used for the prevention of organ transplant rejection and approved by the US Food and Drug Administration (FDA) in September, 1999 for its safety, has been found to synergize with cisplatin in the treatment for basal-like breast cancer cell (22). Moreover, a phase II neoadjuvant endocrine therapy clinical trial demonstrated the synergistic effects of the combination of the mTORC1 inhibitor, everolimus, with letrozole in the treatment of breast cancer (23). However, to the best of our knowledge, there were few studies to date which have investigated whether rapamycin has the potential to enhance the sensitivity of ER⁺ breast cancers to tamoxifen.

In the present study, we found that rapamycin indeed enhanced the sensitivity of ER⁺ breast cancer cells to tamoxifen both *in vitro* and *in vivo*. Moreover, we found that this synergistic effect may be mediated partly through the upregulation of ER expression following the induction of p73. Taken together, combination therapy with rapamycin and tamoxifen may provide new insight and may aid in the development of novel therapeutic strategies for the treatment of ER⁺ breast cancer.

Materials and methods

Cell culture and treatment. The human breast cancer cell lines, MCF-7 and ZR-75-1, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in complete medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM; Wisent Biotechnology, Nanjing, China) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin-streptomycin (HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

For drug treatment, the MCF-7 and ZR-75-1 cells were first treated with tamoxifen (0-25 µM; Sigma-Aldrich, Dorset, UK) or rapamycin (0-6 µM; Sigma-Aldrich) individually for 48 h. Following rapamycin treatment, we found the effective concentration of rapamycin started began from 40 nM with an ~20% inhibitory rate. We selected 40 nM rapamycin to further investigate the effects of the combination of the two drugs on breast cancer. The changes in p73 and ERα expression following rapamycin treatment are shown in Fig. 1A and B.

Cell viability assay. Cell viability was measured using CCK-8 kits (Dojindo, Kumamoto, Japan) following the manufacturer's instruction. Briefly, the MCF7 and ZR-75-1 cells grown in monolayers were harvested and dispensed in 96-well culture plates in 200 µl of DMEM at a concentration of 5x10³ cells per well. After 12 h, the differential drug concentrations of tamoxifen (0-24 µM), rapamycin 40 nM, or both 0-24 µM tamoxifen plus 40 nM rapamycin were added to the cells. After 48 h, the medium in each well was replaced with 100 µl

DMEM containing 10% CCK-8 to measure the growth rate of cells. The plates were incubated at 37°C for 2.5 h and the optical density (OD) values at 450 nm were measured using a microplate reader (Tecan Austria GmbH, Grödig, Austria). Each test was performed in triplicate.

Apoptosis assays. Apoptosis measurements were conducted using the Annexin V apoptosis detection kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Early and late apoptotic, as well as viable cell populations were identified by plotting phycoerythrin (PE), Annexin V vs. 7-AAD (7-amino-actinomycin D). For each measurement, 3 independent samples were pooled.

Drug combination analysis. The combination analysis was conducted using the method previously described by Chou and Talalay (24). Cell viability was measured by CCK-8 assay. Drug dose-effect calculations and the combination indices (CI) for 50% growth inhibition were obtained using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA), and the Student's t test was applied to verify whether the CI values at 50% growth inhibition were significantly different from CI=1. As regards the CI combination indices, CI <1 indicates synergism, CI = 1 indicates additivity, and CI >1 indicates antagonism.

Animal model in vivo. Nude mice were used in this study, 30 female nude mice, aged 4 weeks and weighing 12-15 g, were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, China). The study was approved by the Institutional Animal Care and Use Committee for Animal Use (the Animal Ethics Committee of Nanjing Medical University). The mice were kept under the following conditions: Relative humidity, 40-70%; room temperature, 20-26°C; food and water, 5 g food and 100 ml water per 100 g body weight per day. Estrogen (E2; 0.9 mg/kg) was injected into the abdomen of the 4-week-old female nude mice every 3 days. Subsequently, 5x10⁶ ZR-75-1 cells were injected into the abdominal mammary fat pad of the mice. When the tumor volume reached ~200 mm³, the mice were randomly divided into 4 groups as follows: i) The control group, in which the mice received phosphate-buffered saline (PBS); ii) the rapamycin group, in which the mice received rapamycin (0.25 mg/kg body weight, p.o.); iii) the tamoxifen group, in which mice received tamoxifen (60 mg/kg body weight, p.o.); and iv) the combination group, in which mice received a combination of the two drugs in their drinking water. Tumor growth was measured using a caliper each week. After 4 weeks, the mice were sacrificed and the tumors removed. The excised tumor portions were fixed in 4% paraformaldehyde for further analysis.

Immunohistochemical (IHC) staining and analysis. Breast tissue samples (n=82) were obtained from the First Affiliated Hospital of Nanjing Medical University, China, between 2004 and 2007. The collection and use of the samples was reviewed and approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and informed consent was obtained from all patients prior to sample collection. The TNM staging was defined according to the American Joint Committee on Cancer

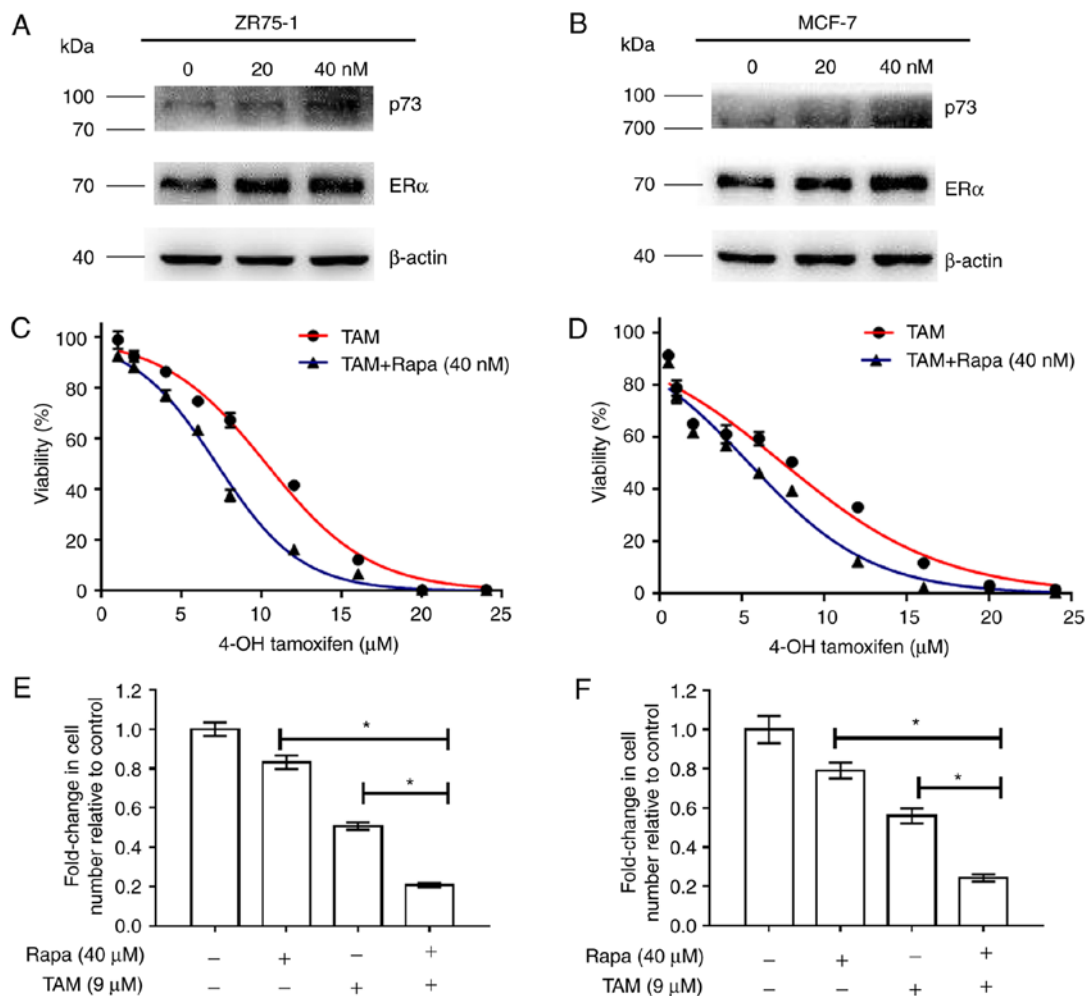


Figure 1. Rapamycin functions synergistically with tamoxifen in the MCF-7 and ZR-75-1 cells. (A and B) Changes in p73 and ERα expression following rapamycin treatment (C and D) MCF-7 and ZR-75-1 cells were treated with tamoxifen at various concentrations (0, 1, 2, 4, 6, 8, 16, 20 and 24 μM), or a combination of tamoxifen and rapamycin (40 nM). After 48 h, cell viability was measured by CCK-8 assays. (E and F) MCF-7 and ZR-75-1 cells were treated with 9 μM tamoxifen, 40 nM rapamycin, or a combination of the two agents for 48 h, and cell viability was measured. Histograms represent the quantification of cell viability. These data were calculated from 3 separate experiments and presented as the means ± SEM, *P<0.05 for the TAM (9 μM) group vs. the TAM (9 μM) + Rapa (40 nM) group. TAM, tamoxifen; Rapa, rapamycin.

(AJCC) (6th version, 2002). IHC staining of the same tissue samples with p73 (diluted 1:50; cat. no. PA5-35368; Thermo Fisher Scientific, Waltham, MA, USA) and ERα antibodies (diluted 1:1,000; cat. no. 13258; Cell Signaling Technology, Danvers, MA, USA) was conducted and analyzed as previously described (25).

Plasmids and siRNA transfection. Plasmids and siRNA constructs of p73 siRNA targeting p73 (siRNA1, siRNA2) and a negative control (CTRi), plasmid targeting p73 and the control (Vector) were obtained from GenePharma (Shanghai, China). Briefly, the MCF-7 and ZR-75-1 cells were transfected with the plasmid or siRNAs using Lipofectamine 3000 (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were cultured in a 6-well plate for 24 to 48 h and the expression level was detected by western blot analysis and reverse transcription-quantitative PCR (RT-qPCR) to determine the transfection efficiency.

Western blot analysis. Western blot analysis was carried out as previously described (26). The radioimmunoprecipitation

assay (RIPA) kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to extract protein from the breast cancer cells according to the manufacturer's instructions. The bicinchoninic Acid Protein Assay kit (BCA) was used to determine the protein concentration. A total of 20 μg of proteins with different molecular weights were separated on 10% SDS-PAGE gels, and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA) in transfer buffer. The membranes were then blocked in 5% non-fat milk at room temperature for ~2 h and incubated in the specific primary antibodies at 4°C overnight. After washing in TBST, the membranes were incubated with secondary antibodies at room temperature for ~2 h. ECL Plus (EMD Millipore) was used to detect the protein bands with the Bio-Imaging System. The following detection antibodies were used: p73 (diluted 1:1,000; cat. no. 14620), ERα (diluted 1:1,000; cat. no. 13258), anti-rabbit secondary antibodies (diluted 1:1,000; cat. no. 7074), anti-mouse secondary antibodies (diluted 1:1,000; cat. no. 7076) (all from Cell Signaling Technology) and β-actin (diluted 1:1,000; cat. no. AA128; Beyotime).

RNA extraction and RT-qPCR. Total RNA was extracted using TRIzol reagent (Takara, Kusatsu, Japan). Reverse transcription and qPCR were performed as previously described (27). The following PCR primers were used to amplify the relevant genes: β -actin forward, 5'-GCTGTGCTATCCCTGTACGC-3' and reverse, 5'-TGCCTCAGCGCAGCGGAACC-3'; p73 forward, 5'-CGGGCCATGCCTGTTTACA-3' and reverse, 5'-TGTCCTTCGTTGAAGTCCCTC-3'; ER α forward, 5'-CCC ACTCAACAGCGTGTCTC-3' and reverse, 5'-CGTCGATTA TCTGAATTTGGCCT-3'. the method of quantification was $2^{-\Delta\Delta C_t}$ (28).

Dual-luciferase reporter assay. Dual-luciferase reporter assays were conducted in triplicate using respective kits (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 200 ng of a pGL3 reporter containing target regions, an internal control, and 5 ng of *Renilla* luciferase vector (pRL-TK; Promega) were co-transfected into the breast cancer cells. After 48 h, the cells were harvested to measure the luciferase activity. All the experiments were conducted at least 3 times.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed using chromatin immunoprecipitation kits (17-371, EZ-ChIP; EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions as described previously (29). The primary antibody used was anti-rabbit p73. An aliquot (2 μ l) of each sample was analyzed by PCR using specific primers listed as follows: Sense, 5'-GCACTTAGAAATGGTCCTGGTAA-3' and antisense, 5'-CCTGCTCAATGACAATCACACT-3'.

Statistical analysis. Each experiment in this study was repeated in triplicate, unless otherwise specified. The data were analyzed using SPSS software (version, 22.0). The association between p73 and the patient clinicopathological parameters was analyzed using χ^2 tests. The correlation between the expression levels of p73 and ER α in the breast cancer specimens was analyzed using a 2-tailed Spearman's correlation analysis. The other data are presented as the means \pm standard error of the mean (SEM) and differences between groups were analyzed using a Student's t-test or ANOVA (with Dunnett's post hoc test). A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Rapamycin sensitizes the ZR-75-1 and MCF-7 cells to tamoxifen in vitro. To examine the effects of the combination of tamoxifen and rapamycin on cell viability, the MCF-7 and ZR-75-1 cells were treated with tamoxifen (0-24 μ M) and/or rapamycin (40 nM) and examined by CCK-8 assay (Fig. 1C and D). As shown in Fig. 1E and F, the inhibitory rates observed with the combination treatment with rapamycin plus tamoxifen reached 83% compared to those observed with treatment with tamoxifen alone (50%) in the ZR-75-1 cells. Likewise, treatment with rapamycin in combination with tamoxifen suppressed cell growth by 79% compared to treatment with tamoxifen alone (55%) in the MCF-7 cells. When the growth inhibition rates reached 50%, the combination indices (CI) achieved were 0.699 and 0.745 in ZR-75-1 and

MCF-7 cells, respectively, suggesting that rapamycin functions synergistically with tamoxifen to inhibit the growth of ER $^{+}$ breast cancer cells (Table I).

Rapamycin enhances the tamoxifen-induced apoptosis of ZR-75-1 and MCF-7 cells. Treatment with rapamycin plus tamoxifen led to a 40% induction of cell apoptosis compared to treatment with tamoxifen alone (19%) in the ZR-75-1 cells (Fig. 2A and C). The apoptotic rate observed with treatment with tamoxifen in combination with rapamycin was 45% compared to treatment with tamoxifen alone (22%) in the MCF-7 cells (Fig. 2B and D). These results demonstrate that rapamycin is capable of functioning synergistically with tamoxifen to enhance the tamoxifen-induced apoptosis of the ZR-75-1 and MCF-7 cells.

Effects of rapamycin and tamoxifen on the growth of ER $^{+}$ breast cancer in vivo. As shown in Fig. 3A and B, compared to the control group, the combination treatment group (rapamycin + tamoxifen) exhibited a significant inhibition of tumor growth by 79.1% at 4 weeks. However, the groups which received rapamycin or tamoxifen alone exhibited a suppression of tumor growth of only 29.9 and 58.8%, respectively, compared with the control group. Furthermore, the tumor weight of the combination treatment group was the lightest of the 4 groups (Fig. 3C). These results indicated that the combination of rapamycin or tamoxifen significantly and synergistically inhibited tumor growth *in vivo*.

IHC staining of p73 and ER α in human breast cancer tissues. As rapamycin is an inducer of p73, we hypothesized that this synergistic effect may be mediated partly through the upregulation of ER expression following the induction of p73. To examine the association between p73 and ER α , IHC staining was performed in 82 breast cancer tissues. As shown in Fig. 4A, p73 was mainly expressed in the cytoplasm and ER α was mainly expressed in the nucleus. Representative images of p73 expression in breast cancer tissues expressing high and low levels of ER α are presented in Fig. 4B. Table II shows the analysis of the association of p73 expression and the clinicopathological characteristics of the breast cancer patients. Additionally, we also found that there was a positive correlation between the expression levels of p73 and ER α in the breast cancer specimens (two-tailed Spearman's correlation analysis, $r = 0.723$, $P < 0.05$) (Fig. 4C). On the whole, these data suggested that ER α expression positively correlated with p73 expression in breast cancer tissues.

p73 regulates ER α expression in MCF-7 and ZR-75-1 cells. To examine whether p73 regulates ER α expression in breast cancer, the MCF-7 and ZR-75-1 cells were transiently transfected with siRNA and scramble siRNA. As shown in Fig. 5C and D, the expression of ER α in the MCF-7 cells was effectively downregulated by siRNA against p73 compared with the cells transfected with the scramble siRNA (CTRi) at both the protein and mRNA level. Moreover, a p73 overexpression plasmid was transfected into the MCF-7 cell lines and the effects of p73 on ER α expression were investigated by RT-qPCR and western blot analysis. As shown in Fig. 5A and B, the expression of ER α was upregulated in the MCF-7 cells transfected with the

Table I. Multiple drug dose-effect calculations and the combination index generated using GraphPad Prism software.

Cell line	RAPA (μ M)	TAM (μ M)	Growth inhibition (%)	CI	Effect	P-value
ZR75-1	3	10.2	50	0.699	Synergy	<0.05
MCF-7	2.5	7.4	50	0.745	Synergy	<0.05

CI, combination indices.

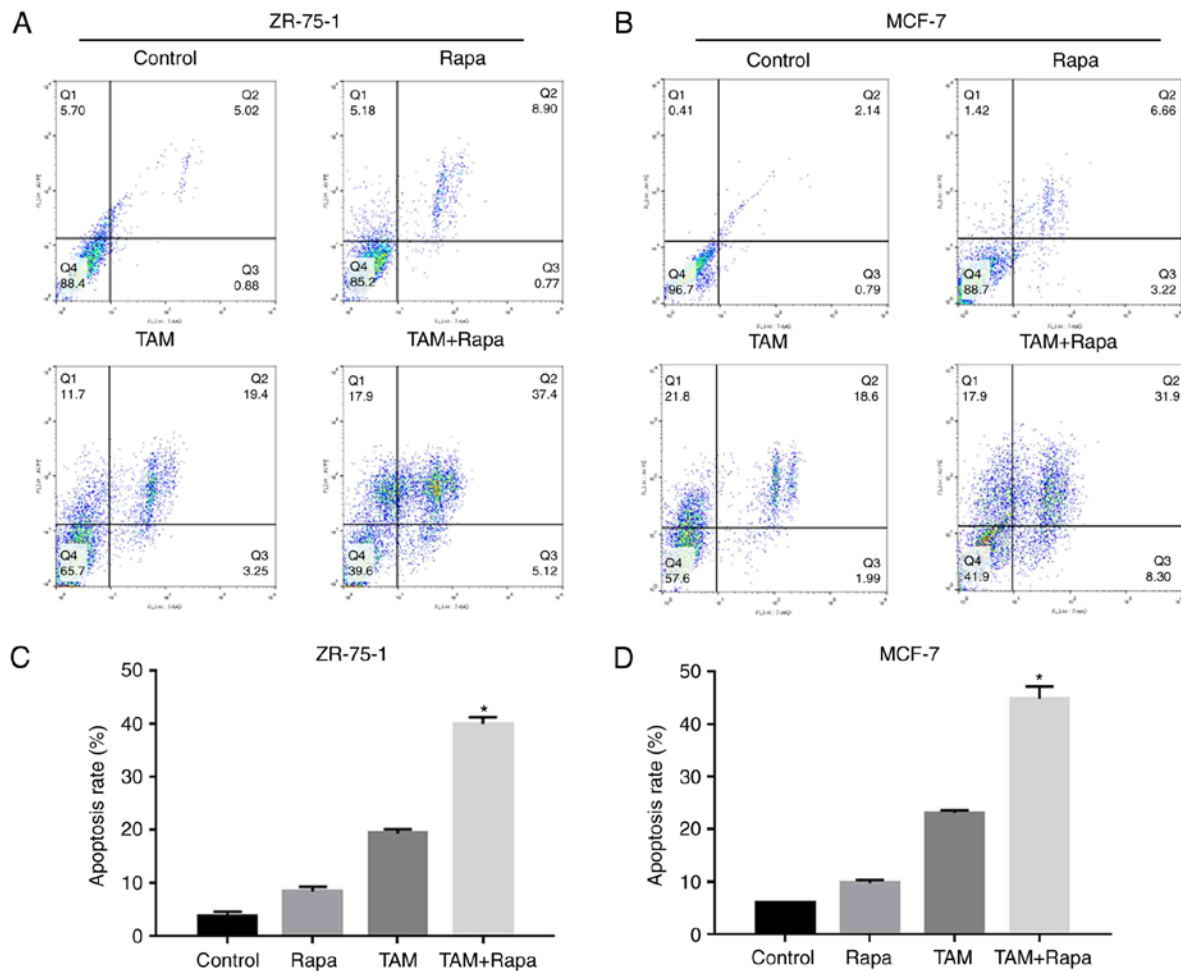


Figure 2. Rapamycin enhances the tamoxifen-induced apoptosis of ZR-75-1 and MCF-7 cells. (A and B) MCF-7 and ZR-75-1 cells were treated with tamoxifen (9 μ M) and/or rapamycin (40 nM) for 48 h. Following treatment, the cells were stained with PE/AnnexinV vs. 7-AAD to examine the apoptosis of the cells. (C and D) Histograms represent the quantification of apoptotic cells. The combination of the two drugs induced more significant apoptotic effects compared to the other groups. These data were calculated from 3 separate experiments and are presented as the means \pm SEM. *P<0.05 for the TAM (9 μ M) group vs. The TAM (9 μ M)+Rapa (40 nM) group. TAM, tamoxifen; Rapa, rapamycin.

p73 overexpression plasmid compared with that of the empty vector-transfected cells. Similar results were also observed in the ZR-75-1 cells (Fig. 5E-H). These data suggest that p73 positively regulates ER α expression in ER⁺ breast cancer cells.

p73 directly binds to the ER α promoter region. To further investigate whether p73 regulates ER α transcription, we hypothesized that p73 regulates the expression of ER α by directly binding to ER α DNA. As shown by the schematic diagram in Fig. 6A, the luciferase reporter constructs contain the E-box in the promoter region of the ER α gene. Thus, the MCF-7 and ZR-75-1 cells

were transfected with a luciferase reporter containing promoter of the ER α gene in order to determine whether p73 directly controls the transcription of the ER gene. The results revealed a 1.6- and 1.3-fold increase in luciferase activity in the MCF-7 and ZR-75-1 cells compared to the control vector-transfected cells, respectively (Fig. 6B and C). Furthermore, ChIP assays revealed that p73 binds to this E-box in the promoter region of ER α in the MCF-7 cells (Fig. 6D). GAPDH, which represented a negative control, was not bound by p73. These data indicate that p73 directly regulates ER α expression by binding to the E-box elements in the promoter region of the ER α gene.

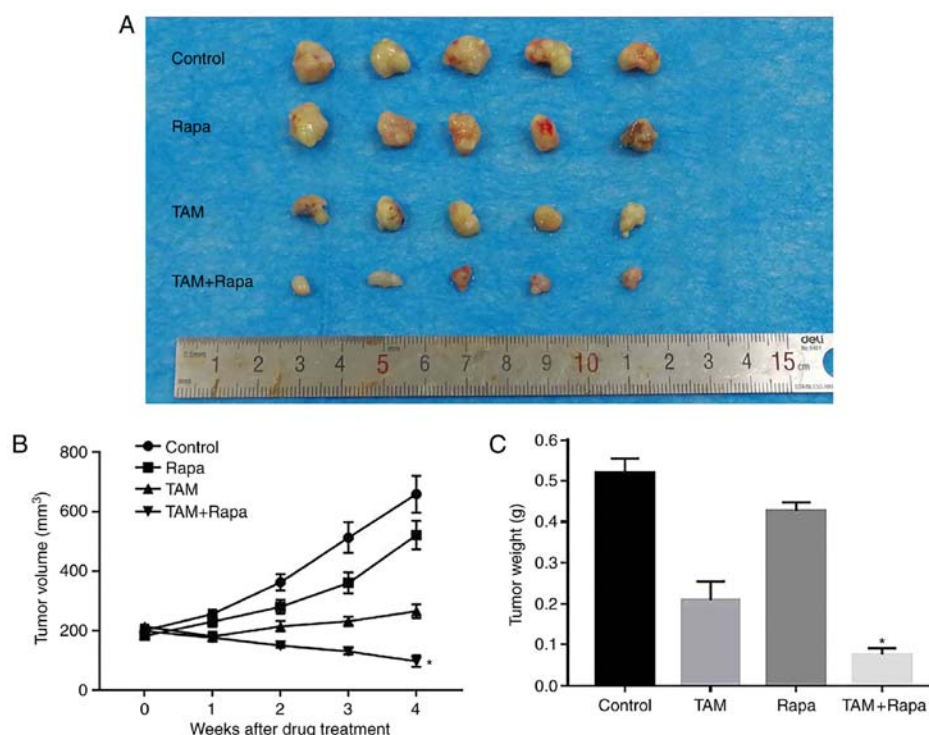


Figure 3. Effects of rapamycin and tamoxifen on the growth of ER+ breast cancer *in vivo*. (A) Images of typical tumors are shown. (B) Tumor growth curve. The mice treated with tamoxifen plus rapamycin formed smaller tumor volume compared to other groups. (C) Histograms represent the weight of tumors from the different groups. The tumor weight of the combination treatment group (TAM + Rapa) was the lightest of the 4 groups. Statistical analysis was performed using one-way ANOVA and the Student's t-test with the day 28 values only. * $P < 0.05$ compared with the TAM group. TAM, tamoxifen; Rapa, rapamycin.

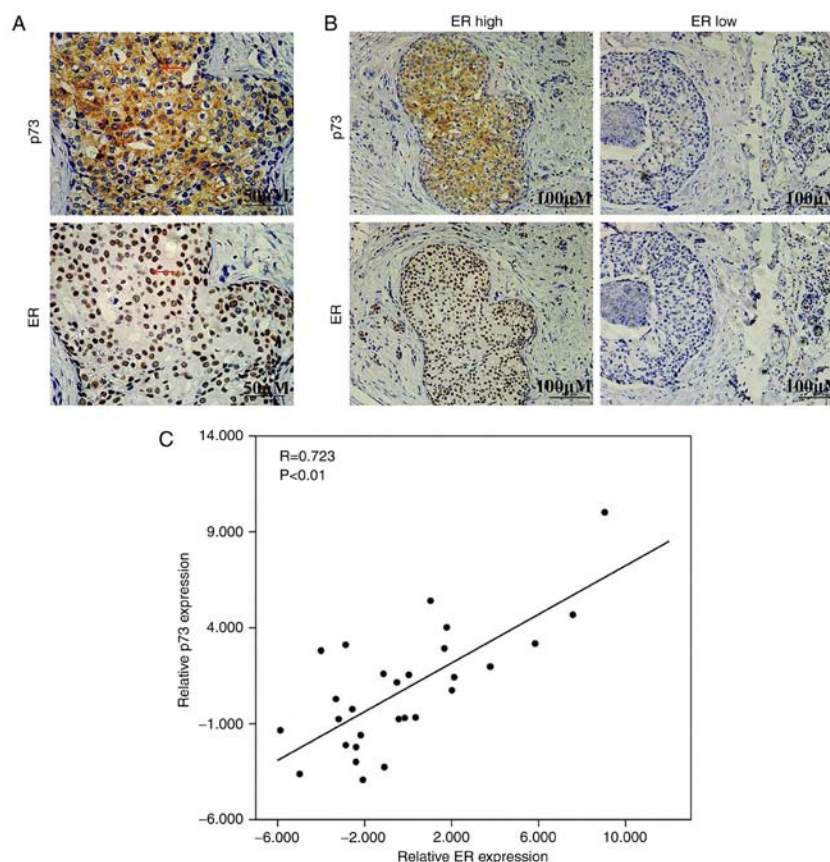


Figure 4. p73 expression is positively associated with ER α in human breast cancer tissues. (A) IHC analysis of p73 and ER α expression in breast cancer at x400 magnification. p73 was mainly expressed in the cytoplasm and ER α was mainly expressed in the nucleus. Scale bars, 50 μ m. (B) IHC analysis of p73 and ER α expression in breast cancer at x200 magnification. Breast cancer tissues with a high expression of ER α expressed high levels of p73; breast cancer tissues with a low expression of ER α expressed low levels of p73. Scale bars, 50 μ m. (C) A positive correlation was observed between the expression levels of p73 and ER α in breast cancer specimens ($P < 0.05$). ER, estrogen receptor.

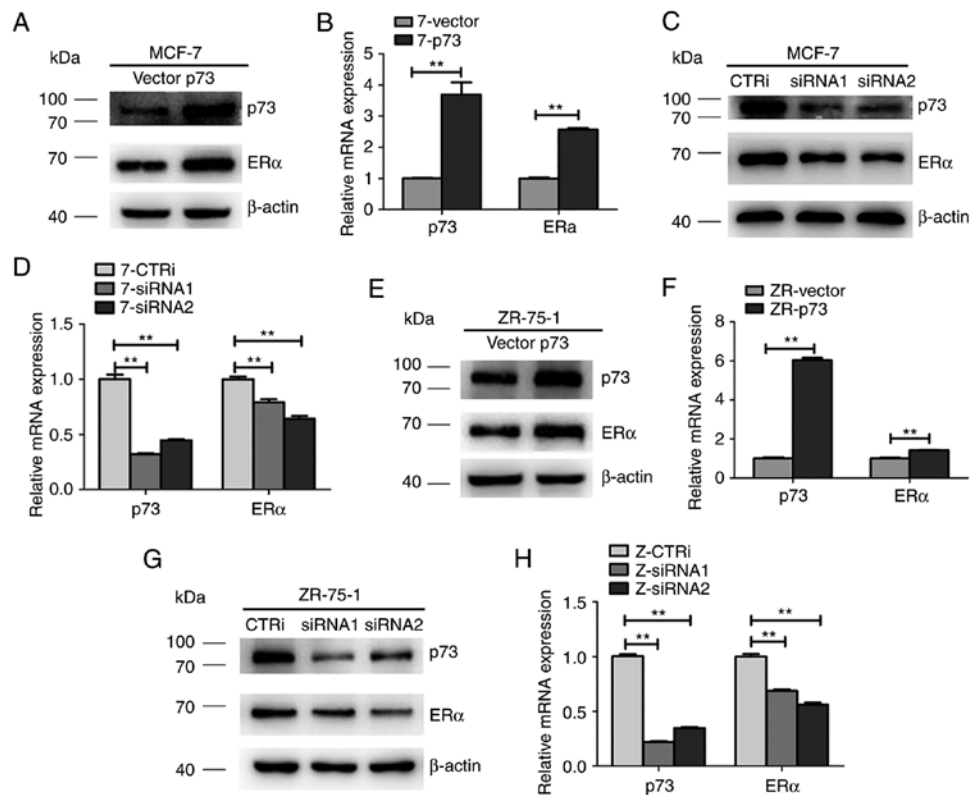


Figure 5. p73 regulates ERα expression in breast cancer cells. (A, B, E and F) MCF-7 and ZR-75-1 cells were transfected with a plasmid to overexpress p73 (p73) and the control (vector). ERα expression was significantly increased at both the protein (A and E) and mRNA level (B and F) following p73 overexpression. (C, D, G and H) MCF-7 and ZR-75-1 cells were transfected with siRNA to knockdown p73 (siRNA1, siRNA2) or control siRNA (CTRi). ERα expression was significantly decreased at both the protein (C and G) and mRNA level (D and H) following p73 knockdown. Western blot analysis and RT-qPCR were performed to detect the expression of p73 and ERα at the protein and mRNA level. Results are representative of 3 independent experiments and are presented as the means ± SEM, **P<0.01. ER, estrogen receptor.

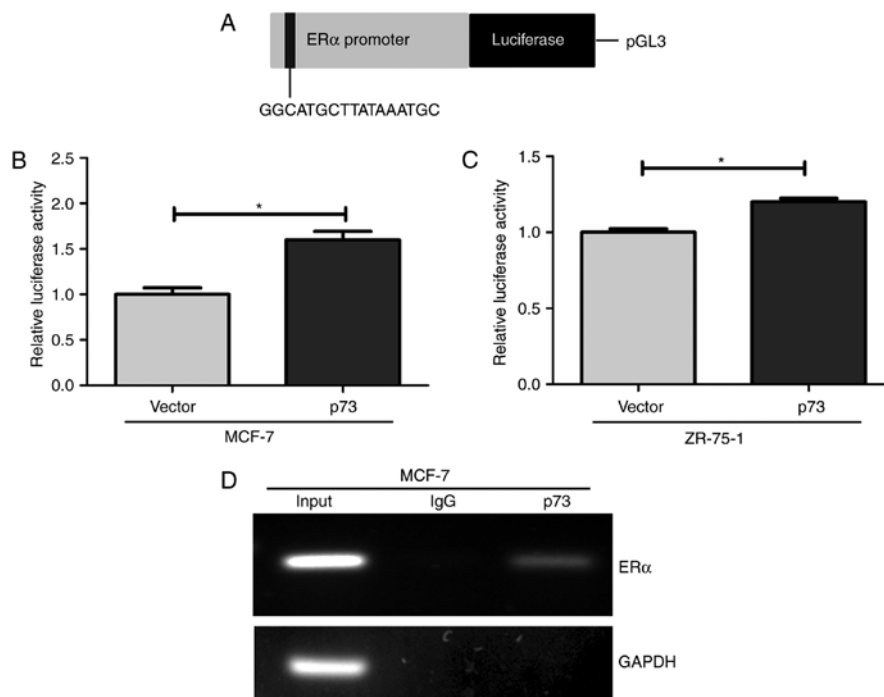


Figure 6. p73 regulates ERα expression by binding to the promoter region of the ERα gene in breast cancer cells. (A) Schematic diagram of the luciferase reporter constructs containing the E-box in promoter region of the ERα gene. (B and C) MCF-7 and ZR-75-1 cells transfected with the p73 overexpression plasmid or the control were transfected with the pGL3 control reporter or pGL3 reporter carrying the E-box in promoter region of the ERα gene. The increased fold in relative luciferase activity was a product of the luciferase activity induced by p73 overexpression divided by that induced by the control. Results are representative of 3 independent experiments and are presented as the means ± SEM. *P<0.05. (D) p73 directly binds to E-box in promoter region of the ERα gene. Lane 1, input DNA; lane 2, DNA from MCF-7 cells immunoprecipitated with normal mouse IgG; lane 3, DNA from MCF-7 cells immunoprecipitated with anti-p73 antibody. ER, estrogen receptor.

Table II. Association of p73 with ER α and clinicopathological characteristics of breast cancer patients.

Clinicopathological characteristics	p73 expression			P-value
	No. of cases	Low (%)	High (%)	
Age, years				0.247
<50	35	26 (74.3)	9 (25.7)	
\geq 50	47	29 (61.7)	18 (37.9)	
Pathological grade				0.482
I-II	43	27 (62.8)	16 (37.2)	
III	39	28 (71.8)	11 (28.2)	
TNM stage				0.983
I-II	76	51 (67.1)	25 (32.9)	
III	6	4 (66.7)	2 (33.3)	
Tumor size (cm)				0.459
\leq 2	28	17 (60.7)	11 (39.3)	
>2	54	38 (70.4)	16 (29.6)	
Lymph node metastasis				0.920
N0	51	34 (66.7)	17 (33.3)	
N1-N3	31	21 (67.7)	10 (32.3)	
ER				0.019
Negative	46	36 (78.3)	10 (21.7)	
Positive	36	19 (52.8)	17 (47.2)	
PR				0.945
Negative	68	46 (67.6)	22 (32.4)	
Positive	14	9 (64.3)	5 (35.7)	
Her2				0.232
Negative	33	25 (75.8)	8 (24.2)	
Positive	49	30 (61.2)	19 (38.8)	

ER, estrogen receptor; PR, progesterone receptor.

Discussion

In the present study, we found that the mTOR inhibitor, rapamycin, which has been approved by the FDA for the prevention of organ transplant rejection, enhances the sensitivity of ER⁺ breast cancer cells to tamoxifen partly through the upregulation of ER expression following the induction of p73.

In patients with metastatic breast cancers, tamoxifen treatment would lead to disease regression in ~30% of cases (30). Patients who receive tamoxifen treatment for 0 to 4 years obtain maximal benefits, with a reduced recurrence rate 51% and a reduced death rate by 28%. The reduction in recurrence and mortality is sustained in year 5 and beyond (30,31). In a recent study, the worldwide Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial, indicated that 10 years of tamoxifen treatment reduced breast cancer recurrence and mortality more effectively than treatment for 5 years (32). This fully affirmed the efficacy of tamoxifen in the treatment for ER⁺ breast cancer, while its severe side-effects following long-term and high-dose treatment must still be seriously considered. In this study, we observed the synergistic effects of tamoxifen and rapamycin used in combination in ER⁺ breast cancer cell

lines. In addition, this synergistic effect of rapamycin and tamoxifen was confirmed using a nude mouse model *in vivo*. The dose of rapamycin (40 nM) used in combination with tamoxifen was lower than that of metformin (5 mM) and celecoxib (30 μ M) used in previous studies (19,20). This indicates that rapamycin may be a more desirable drug for the prevention of breast cancers and the growth inhibition of existing tumors in women. To date, apart from breast cancer, the combination of rapamycin and CC-5013 (Revlimid) has been shown to improve patient outcome in multiple myeloma (33). Of note, in this study, the concentration of tamoxifen required for growth inhibition was substantially reduced when rapamycin was combined with tamoxifen.

The mTOR pathway plays a crucial role in multiple cellular processes and is the most frequently activated signaling pathway, which promotes tumor growth and progression (34). Gene alterations in the mTOR pathway are frequently observed in ER⁺ breast cancer. These include insulin-like growth factor 1 receptor (IGF-1R), phosphatidylinositol 3-kinase (PI3K) and human epidermal growth factor receptor 2 (HER2) gene amplifications (35-37) or phosphatase and tensin homolog (PTEN) gene function loss (38). Correspondingly, the loss of PTEN

expression is associated with low ER α levels and high PI3K activity, which may result in a poor response to tamoxifen treatment (39,40). Hence, the hyperactivation of the mTOR pathway may lead to the downregulation of ER α expression and may promote hormone-independent cell growth. mTOR inhibitors (e.g., rapamycin) may reverse this process by increasing ER α levels, thereby restoring hormone dependence and sensitivity to endocrine therapy (41).

A crosstalk exists between the mTOR pathway, and ER α and p53-family members. p53 has been reported to regulate ER α expression through transcriptional control by binding to the ER α promoter (42). p73 is structurally and functionally related to p53; however, to date, at least to the best of our knowledge, there are no studies available on the interaction between p73 and ER α . Additionally, rapamycin can selectively increase p73 occupancy at its binding sites and modulate its activity and function (43). As both ER and p73 are involved in the mTOR pathway, we hypothesized that the activation of p73 may regulate ER α expression. In this study, IHC and tissue microarray analysis were applied to confirm the association between p73 and ER α . Moreover, the upregulation of p73 resulted in an increased ER α mRNA and protein expression, whereas the knockdown of p73 decreased the levels of ER α protein and transcript in the ER $^{+}$ breast cancer cells. These data suggest that the expression of p73 and ER α is linked in ER $^{+}$ breast cancer, which would be expected to account for the synergistic effects of rapamycin plus tamoxifen.

The combination of tamoxifen and rapamycin, though, has not been previously investigated in clinical trials, at least to the best of our knowledge. However, was previously reported that the mTORC1 inhibitor, everolimus, plus tamoxifen increased the 6-month clinical benefit rate by 61% compared to 42% with tamoxifen alone and reduced the risk of death by 55% in women with metastatic breast cancer. Moreover, the progression time appeared to be more prolonged with the combination vs. tamoxifen alone (8.6 months vs. 4.5 months, hazard ratio 0.54) (44).

In conclusion, in the present study, we revealed a novel mechanism in that p73 transcriptionally regulates ER α expression by directly binding in its promoter region. In addition, combination therapy with an mTOR inhibitor and tamoxifen, leading to the activation of p73 may provide new insight and may aid in the development of novel strategies for the treatment of patients with ER $^{+}$ breast cancers.

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Availability of data and materials

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

Authors' contributions

QD, JW, JFW and LZ contributed to the design of this study. XXL, LS and JW contributed to the experimental work. JYQ, TSX and WBZ contributed to the data collection and analysis. XS, XJZ contributed to the interpretation of the data and the drafting of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The collection and use of the samples was reviewed and approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and informed consent was obtained from all patients prior to sample collection. The use of animals in this study was approved by the Institutional Animal Care and Use Committee for Animal Use (the Animal Ethics Committee of Nanjing Medical University).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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