

Lapatinib induces p27^{Kip1}-dependent G₁ arrest through both transcriptional and post-translational mechanisms

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Abbreviations: HER2, human epidermal growth factor receptor 2; TKIs, tyrosine kinase inhibitors; CDKs, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitors; Ser, serine; Thr, threonine; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein; DYRK1B, dual-specificity tyrosine-phosphorylation regulated kinase 1B; qPCR, quantitative real-time PCR; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FOXO3a, Forkhead box O3a; CHX, cycloheximide

Lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor, has been shown to have potent antitumor effects against human breast cancer. Recent studies have shown that lapatinib upregulates p27^{Kip1} (here after referred to as p27) expression and induces G₁ cell cycle arrest in various types of cancer cells. However, the regulation of p27 in lapatinib-induced cell cycle arrest is not well studied. Here we demonstrate that lapatinib-induced cell growth inhibition and G₁ cell cycle arrest in HER2-overexpressing human breast cancer cells were dependent on p27. We also show that lapatinib-induced upregulation of p27 expression was mediated through both transcriptional and post-translational mechanisms. On the one hand, lapatinib treatment led to increased FOXO3a expression and enhanced p27 transcription. On the other hand, lapatinib treatment resulted in increased DYRK1B expression, which correlated with increased p27 phosphorylation at Ser10 and decreased p27 degradation. Interestingly, we found that ERβ1 but not ERβ2 expression also upregulated p27 and enhanced lapatinib-induced cell proliferation inhibition and G₁ cell cycle arrest in HER2-overexpressing breast cancer cells. Taken together, our results suggest that lapatinib induces p27 expression via both transcriptional and post-translational upregulations, leading to cell cycle arrest and cell proliferation inhibition, and that its effect on breast cancer cells may be modified by ER expression status.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458 400) of the total cancer deaths in 2008.¹ In addition to early diagnosis, development of new therapies will be important to improve clinical outcome of breast cancer. In recent years, many efforts have been focused on the research of targeted therapy. Among the most studied molecular targets, the human epidermal growth factor receptor 2 (HER2) is a potent mediator of cellular growth and proliferation.^{2,3} HER2 gene overexpression occurs in approximately 20–30% of metastatic breast cancers and is associated with aggressive disease and decreased survival.^{4,5} Since HER2 plays important

roles in breast cancer, several therapeutic approaches have been developed to target HER2, including monoclonal antibodies and small molecule TK inhibitors (TKIs). The humanized monoclonal antibody trastuzumab (Herceptin) has improved disease-free and overall survival in patients with both metastatic and early HER2-positive breast cancer.⁶ Lapatinib (GW572016, GlaxoSmithKline) is a dual EGFR/HER2 tyrosine kinase inhibitor that prevents downstream cell proliferation and survival signaling via the PI3K/AKT and Erk/MAPK pathways.⁷ Recent Phase III clinical studies have shown that lapatinib improves progression-free survival in patients who have progressed on trastuzumab and is approved for treatment of patients with advanced HER2-positive breast cancer in combination with capecitabine.⁸

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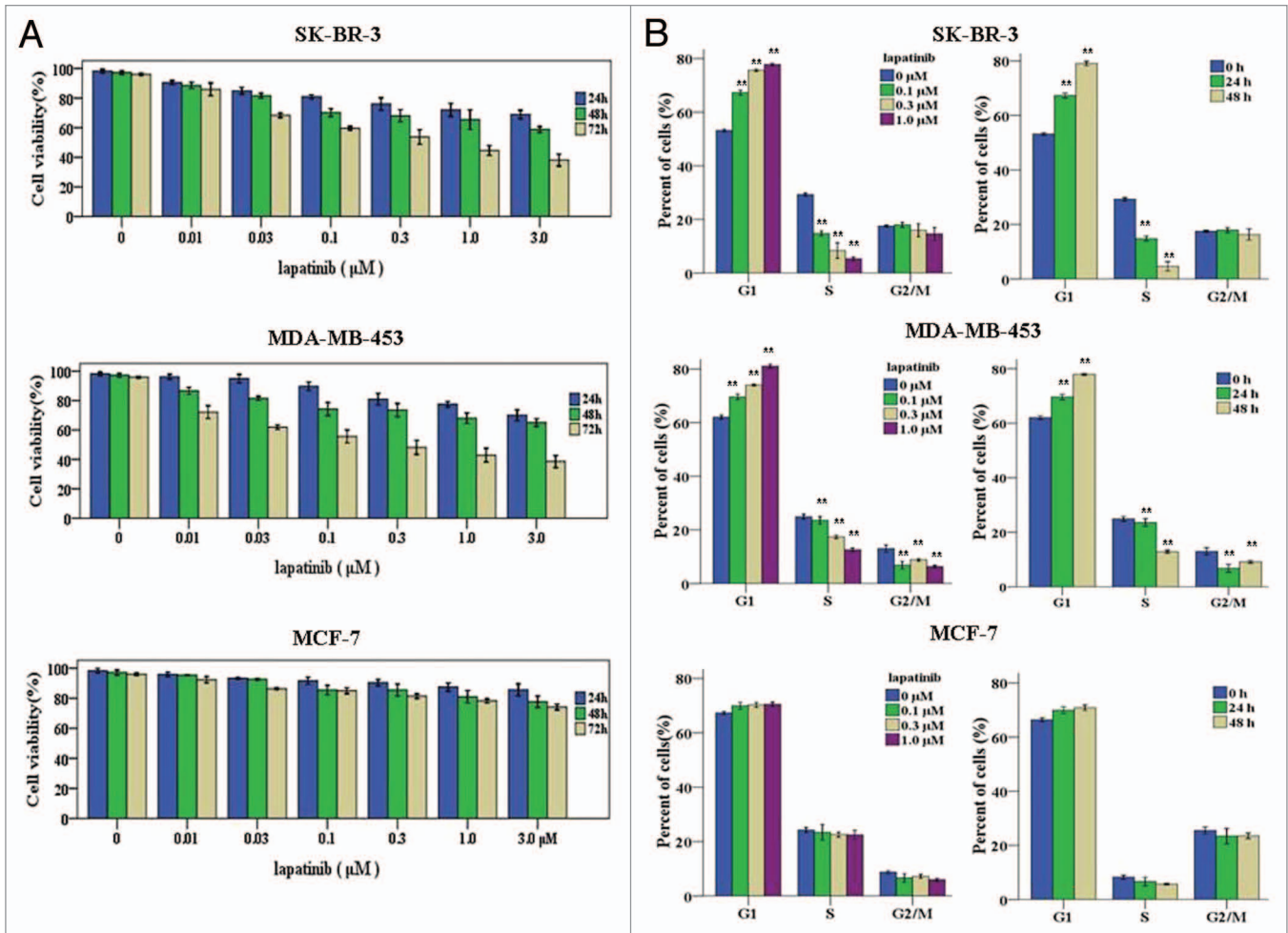


Figure 1. Lapatinib inhibits cell proliferation and causes G₁ cell cycle arrest in EGFR+/HER2+ breast cancer cell lines. (A) SK-BR-3, MDA-MB-453, and MCF-7 cells were treated with various concentrations of lapatinib for indicated time, and MTT assay was performed to determine the cell viability. (B) SK-BR-3, MDA-MB-453, and MCF-7 cells were exposed to various concentrations of lapatinib for 24 h, or to 0.1 μM lapatinib for indicated time, and cell cycle distribution was determined by flow cytometry analysis, ***P* < 0.01.

Previous studies have shown that lapatinib-mediated suppression of cancer cell proliferation correlates with G₁ phase cell cycle arrest or apoptosis induction in vitro and in vivo.^{9,10} Many molecules are involved in cell cycle progression in eukaryotic cells, including a series of protein complexes composed of cyclins, cyclin-dependent kinases (CDKs), and their cyclin-dependent kinase inhibitors (CKIs). p27 is a member of the Cip/Kip family of CDK inhibitory proteins, which plays a pivotal role in the control of cell proliferation, differentiation, and apoptosis.¹¹ p27 is a negative regulator of the protein kinase CDK2/cyclin E and can block the cell cycle at G₀/G₁ phase.¹² In quiescent cells, p27 exhibits maximal translation and stability and contributes to blocked transition from G₁ phase to S phase of the cell cycle via inhibition of cyclin-CDK complexes.^{13,14} Upon mitogenic stimulation, the level of p27 protein decreases rapidly, thus allowing the activation of cyclin E/CDK2 and cyclin A/CDK2 complexes to promote cell proliferation.¹⁵ Forced expression of p27 results in cell cycle arrest in G₁ phase; conversely, inhibition of p27 expression increases the number of cells in S phase. Thus, p27 is a major regulator of G₁/S transition in the cell cycle. Numerous studies

had indicated that p27 protein levels may be associated with the development and survival of human cancers and seem to be an important marker of cancer progression.^{16,17} In addition, several studies have demonstrated that regulation of p27 might be one of the mechanisms through which lapatinib blocks cell cycle progression in cancer cells.^{9,18,19}

Estrogen is essential for development and growth of mammary gland and has been associated with the proliferation and progression of breast cancer. The expression of estrogen receptors (ER) α and β plays an important role in cell cycle regulation. ERα is believed to regulate expression of genes involved in cell survival and proliferation, thus promoting tumor growth and differentiation, while the function of ERβ has been found to be anti-proliferative and pro-apoptotic.²⁰⁻²³ ERβ has several splicing variants, including ERβ1 (wild type), ERβ2 (identical to ERβcx), ERβ3, ERβ4, ERβ5, and ERΔ5.^{24,25} Interestingly, ERβ1 has been shown to act as a tumor suppressor,²⁶ and its expression declines during breast cancer progression.^{27,28} Furthermore, ERβ1 inhibits epithelial-mesenchymal transition (EMT) and invasion in basal-like breast cancer cells.²⁹ ERβ2 appears to be a

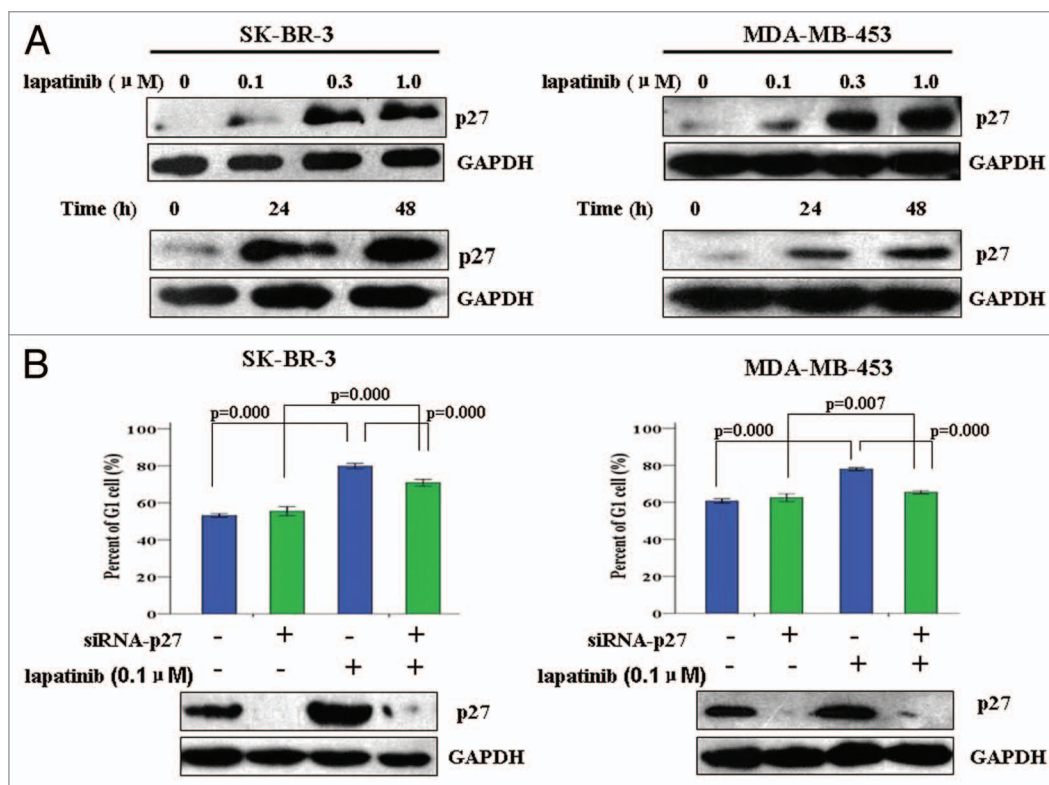


Figure 2. Lapatinib-induced G_1 arrest in EGFR+/HER2+ breast cancer cell lines is dependent on p27. (A) SK-BR-3 and MDA-MB-453 cells were treated with various concentrations of lapatinib for 24 h, or 0.1 μ M lapatinib for indicated time, and p27 protein levels were determined by western blot. (B) SK-BR-3 and MDA-MB-453 cells were transfected with control or p27 siRNA for 48 h, and treated with DMSO or 0.1 μ M lapatinib for additional 24 h. Western blot was performed to determine the efficiency of p27 knockdown, and cell cycle distribution was quantified by flow cytometry analysis.

powerful prognostic indicator in breast cancer, and nuclear and cytoplasmic expression differentially affect outcome. Nuclear ER β 2 immunoreactivity was shown to be significantly associated with better overall survival, while cytoplasmic ER β 2 was significantly associated with high-grade tumors.²⁸ Recently, it was shown that ER α blockade effectively arrested breast cancer cells at G_1 cell cycle by stabilizing p27.^{30,31} However, the roles of ER β 1 and ER β 2 in this process have not yet been clearly established.

Several groups have demonstrated that lapatinib induces p27 in breast cancer cells.^{32,33} However, how p27 expression is regulated after lapatinib treatment is not fully understood. In this study, we found that p27 is required for the G_1 cell cycle arrest effect of lapatinib in HER2-overexpressing breast cancer cells. We also established that lapatinib-induced p27 expression regulation is at both transcriptional level via promoter activation and post-translational level via Ser10 phosphorylation, which prevents protein degradation. Interestingly, we also found that ER β 1 and ER β 2 expression status may effect p27 expression and modify the effect of lapatinib.

Results

Lapatinib inhibits cells proliferation and causes G_1 phase cell cycle arrest in HER2-overexpressing breast cancer cells

To investigate the effects of lapatinib on cellular proliferation, we analyzed the inhibitory effects of increasing concentrations

of lapatinib on SK-BR-3, MBA-MD-453, and MCF-7 cells by MTT assay. These human breast cancer cells were exposed to various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 μ M) of lapatinib for different time durations (24, 48, or 72 h), and cell survival percentages were determined. Inhibition of the proliferation of SK-BR-3 and MBA-MD-453 cells by lapatinib treatment was readily detected, which was clearly time- and dose-dependent (Fig. 1A). However, there was no significant effect of lapatinib on the growth of MCF-7 cells, which are HER2-negative (Fig. 1A).

To determine the potential mechanisms by which lapatinib inhibits human breast cancer cell growth, the effect of lapatinib on cell cycle progression was evaluated by flow cytometry. When SK-BR-3 and MBA-MD-453 cells were treated with lapatinib at different doses (0.1, 0.3, 1 μ M) for 24 or 48 h, G_1 cell cycle arrest was induced in a time- and dose-dependent way (Fig. 1B). In contrast, treatment of MCF-7 cells with lapatinib did not induce discernible changes in cell cycle distribution (Fig. 1B). Taken together, these results showed that lapatinib could cause G_1 phase cell cycle arrest and therefore inhibit cell proliferation in HER2-overexpressing breast cancer cells.

Lapatinib-induced G_1 arrest in HER2-overexpressing breast cancer cell lines is dependent on p27

To understand how lapatinib induces G_1 cell cycle arrest, we examined the expression and function of p27 in G_1/S transition. To this end, SK-BR-3 and MBA-MD-453 breast cancer cells were treated with various concentrations of lapatinib for 48 h, and p27

expression was assessed. As shown in **Figure 2A**, lapatinib induced p27 expression in a dose-dependent manner. Similarly, when the cells were exposed to 0.1 μM lapatinib for different duration, a time-dependent p27 upregulation was observed (**Fig. 2A**).

To determine whether lapatinib-induced upregulation of p27 expression is required for its G_1 cell cycle arrest effect, we depleted p27 from SK-BR-3 and MBA-MD-453 cells and asked whether this would abolish lapatinib's effect in cell cycle regulation. As shown in **Figure 2B**, when the cells were transfected with p27-targeting siRNA, p27 expression was efficiently suppressed, and lapatinib-induced G_1 cell cycle arrest was also inhibited. Collectively, these results strongly suggest that lapatinib-induced G_1 arrest in HER2-overexpressing breast cancer cell lines is dependent on its upregulation of p27.

Lapatinib increases promoter activity and mRNA levels of p27 in SK-BR-3 cells

Next we sought to determine how lapatinib regulates p27 expression. We first examined whether lapatinib treatment increased p27 transcription. To this end, we exposed SK-BR-3 cells to 0.1 μM lapatinib for 48 h, and assessed p27 mRNA levels by quantitative RT-PCR. As shown in **Figure 3A**, lapatinib treatment led to a 3.4-fold increase of p27 transcripts in SK-BR-3 cells, suggesting that lapatinib may enhance p27 transcription. To confirm whether this is the case, we used a luciferase reporter assay to assess

the p27 promoted activity before and after lapatinib treatment. We transfected a p27 promoter driven luciferase reporter construct into SK-BR-3 cells and treated the cells with 0.1 μM lapatinib. As shown in **Figure 3B**, 48 h of lapatinib treatment resulted in significantly enhanced p27 promoter activity, as reflected by markedly increased luciferase expression. These data suggest that lapatinib potentially upregulates the transcription of p27.

Previous studies have shown that the expression of FOXO3a (forkhead transcription factor in rhabdomyosarcoma-like1), which is one of the principal regulators of p27 gene transcription, correlated with that of p27.^{34,35} To investigate whether lapatinib enhances transcription of p27 by upregulating FOXO3a expression, we examined FOXO3a mRNA and protein levels upon lapatinib treatment. As shown in **Figure 3C and D**, lapatinib significantly upregulates FOXO3a expression, as reflected by increased mRNA level detected by quantitative RT-PCR and protein level detected by western blot. Collectively, these results suggest that lapatinib enhances p27 transcription by upregulating FOXO3a, one of the principle transcription factors that control p27 expression.

Lapatinib induces p27 phosphorylation at Serine10 and inhibits its degradation

To understand whether lapatinib also confers p27 expression regulation at post-translational level, we first measured the protein

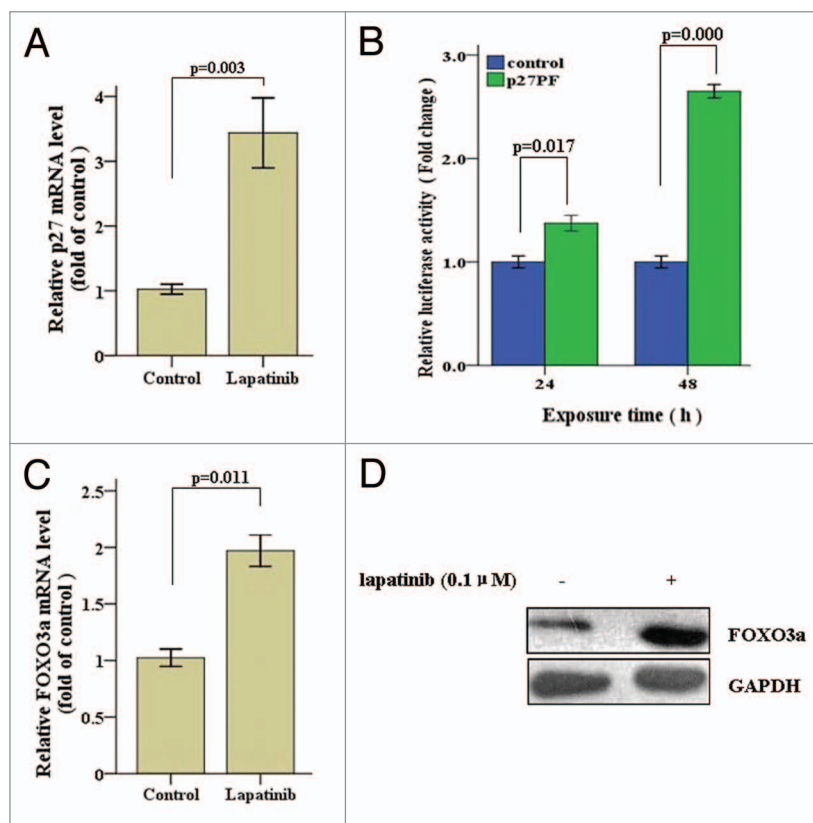


Figure 3. Lapatinib treatment upregulates p27 transcription in SK-BR-3 cells. **(A)** SK-BR-3 cells were treated with 0.1 μM lapatinib for 48 h, and p27 mRNA levels were determined by qRT-PCR. **(B)** SK-BR-3 cells were transfected with a p27 promoter driven firefly luciferase construct (p27PF) as well as a β -galactosidase (β -Gal) plasmid (as internal control). Twelve hours after transfection, cells were treated with 0.1 μM lapatinib for 24 or 48 h, and assayed for luciferase activity. **(C)** SK-BR-3 cells were exposed to 0.1 μM lapatinib for 48 h, and FOXO3a mRNA levels were determined by qRT-PCR. **(D)** SK-BR-3 cells were exposed to 0.1 μM lapatinib for 48 h, and FOXO3a protein levels were determined by western blot.

half-life of p27 in the absence and presence of lapatinib. SK-BR-3 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) together with DMSO or lapatinib and harvested at different time points, and the dynamics of p27 protein levels were measured by western blot. As shown in **Figure 4A**, SK-BR-3 cells treated with lapatinib showed delayed p27 degradation compared with those treated with DMSO. When p27 expression was normalized against GAPDH (internal control), the half-life of p27 in lapatinib-treated cells was calculated to be ~7.0 h, whereas the half-life of p27 in DMSO-treated cells was ~2.5 h (**Fig. 4B**). These data suggest that lapatinib significantly prolongs the half-life of p27 protein by inhibiting its degradation.

Phosphorylation of p27 protein has been widely recognized to be one of the major post-translational mechanisms that regulate the abundance of this protein.³⁶ To determine whether lapatinib regulates p27 phosphorylation, we exposed SK-BR-3 cells to 0.1 μ M lapatinib, and found that lapatinib treatment increased p27 phosphorylation at Ser10 but decreased its phosphorylation at Thr187. Phosphorylation of p27 at Thr187 has been shown to enhance p27 degradation.^{37,38} Therefore, decreased Thr187 phosphorylation is associated with increased p27 stability. The effect of Ser10 phosphorylation on p27 stability is complicated by different kinases, including DYRK1B and KIST, which inhibit and enhance p27 degradation, respectively.^{39,40} We found that upon

lapatinib treatment, DYRK1B mRNA and protein expression was significantly upregulated (**Fig. 5A and B**). In contrast, the mRNA and protein levels of KIST were not significantly reduced (**Fig. 5A and B**). These data suggest that lapatinib may regulate DYRK1B, but not KIST, expression and p27 phosphorylation at Ser10, thereby affecting its stability.

To further determine whether DYRK1B expression and p27 phosphorylation at Ser10 are involved in lapatinib-induced G₁ cell cycle arrest, we transfected SK-BR-3 cells with a DYRK1B-expressing plasmid, treated the cells with 0.1 μ M lapatinib for 48 h, and examined the cell cycle distribution. We found that overexpression of DYRK1B (**Fig. 5C**) led to a further increase of cell population at G₁ phase (**Fig. 5D**), suggesting that p27 phosphorylation at Ser10 by DYRK1B enhances its stability and leads to more efficient cell cycle arrest at G₁ phase.

ER β 1 and ER β 2 affect p27 expression and lapatinib-induced G₁ cell cycle arrest

ER β 1 and ER β 2 have opposing roles in regulating proliferation and bone metastasis genes in the prostate cancer cell line⁴¹ and seem to have different effects on breast cancer cell proliferation.^{42,43} We asked whether this is also the case in our system, and investigated whether this affects cellular response to lapatinib. To this end, we transiently transfected SK-BR-3 cells with ER β 1- or ER β 2-expressing vectors, and treated them with 0.1

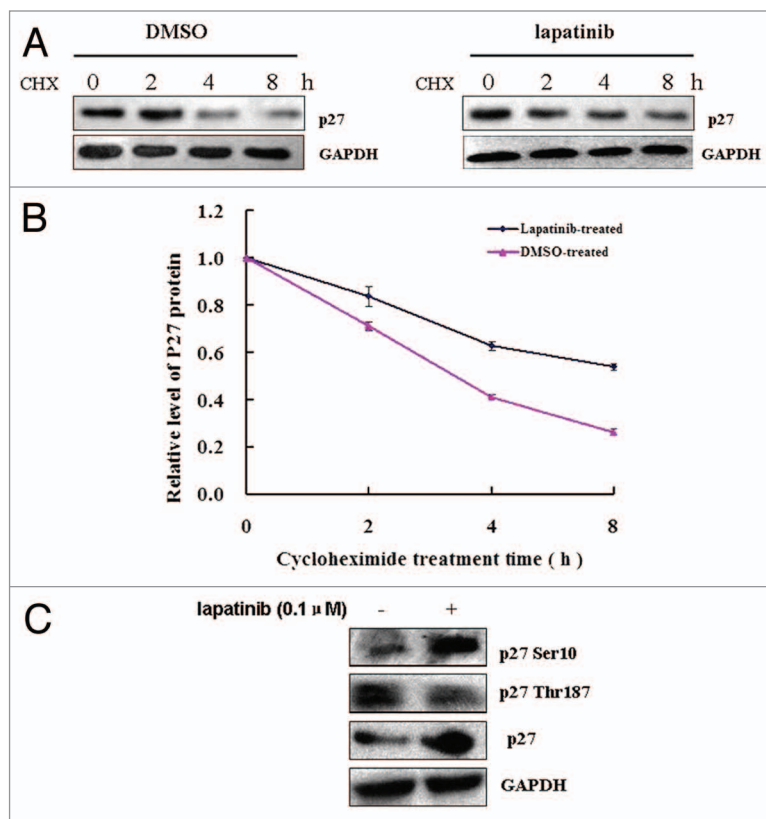


Figure 4. Lapatinib treatment promotes p27 phosphorylation at Serine10 and inhibits p27 degradation in SK-BR-3 cells. **(A)** SK-BR-3 cells were treated with 0.1 μ M lapatinib or DMSO for 48 h, washed, and treated with 5 μ g/mL of cycloheximide (CHX) for the indicated time, and p27 protein levels were determined by western blot. Representative blots from multiple experiments are shown. **(B)** Quantification of p27 protein levels as normalized to the levels of GAPDH. **(C)** SK-BR-3 cells were treated with 0.1 μ M lapatinib for 48 h, and p27 phosphorylation at Serine 10 and Threonine 187 was determined by western blot.

μM lapatinib for 48 h. We found that ER β 1 expression inhibited cell proliferation and enhanced G₁ cell cycle arrest, both in the presence and absence of lapatinib (Fig. 6A and B). However, ER β 2 expression increased cell proliferation, although it did not seem to alter cell cycle distribution (Fig. 6A and B). In addition, we found that ER β 1 expression increased p27 expression, while ER β 2 did not affect p27 protein levels (Fig. 6C). These results suggest that ER β 1 but not ER β 2 expression could regulate p27 expression, and ER expression status may affect lapatinib-induced p27 expression and cell cycle arrest.

Discussion

Lapatinib has been shown to induce cell cycle arrest in many cancer cells. It also induces p27 expression, but mechanisms underlying p27 expression regulation remain unclear. In the present study, we confirmed that lapatinib could induce G₁ cell cycle arrest and upregulation of p27 expression in HER2-overexpressing breast cancer cells. Importantly, we demonstrated that the lapatinib-induced G₁ arrest was dependent on p27, suggesting that regulation of p27 expression is a key mechanism in lapatinib causing cell cycle arrest and cell growth inhibition. In addition, we found that lapatinib regulates p27 expression at both transcriptional and post-translational levels. Moreover, we demonstrated that expression of ER β 1 also regulates p27 expression and affects cell cycle progression. Collectively, our study provides insights into regulation of p27 expression in breast cancer cells by targeted therapy as well as hormone receptors (Fig. 7).

Both promoter activity and mRNA level of p27 were increased upon lapatinib treatment of SK-BR-3 cells. Lapatinib may exert its effects on p27 transcription through multiple transcription factors, including forkhead transcription factor, c-myc, E2F, SP1, and NF-Y,⁴⁴⁻⁴⁶ which are all known to bind directly to the p27 promoter. In our study, we found that lapatinib treatment increased FOXO3a expression, indicating that FOXO3a may mediate the activation of p27 promoter in this setting. However, whether other transcription factors are also involved in the lapatinib-induced transcriptional regulation of p27 needs to be elucidated. A possible approach to identify the transcription factors responsible for lapatinib induced p27 transcription activation would be through microarray analysis. Understanding which transcription factors are altered by lapatinib treatment not only could help to further elucidate the detailed transcription regulation of p27 by this drug, but also to explore other mechanisms through which lapatinib exerts its cell cycle and cell proliferation regulation.

We demonstrated that lapatinib increased the half-life of p27 protein by regulating its phosphorylation, thereby inhibiting its degradation. Thr187 and Ser10 are 2 major sites of p27 phosphorylation that regulate p27 subcellular localization, stability, and function in the cells.⁴⁷ Lapatinib is able to decrease p27 phosphorylation at Thr187 and increase p27 phosphorylation at Ser10. The process of p27 protein degradation partially depends on phosphorylation at Thr187 by cyclin E/CDK2 complex, which binds to ubiquitin ligase SCF^{Skp2}, leading to proteasome degradation.^{48,49} Therefore, by reducing phosphorylation of p27 protein at Thr187, lapatinib could stabilize p27 in SK-BR-3 cells. Phosphorylation of Ser10 also contributes to regulation of

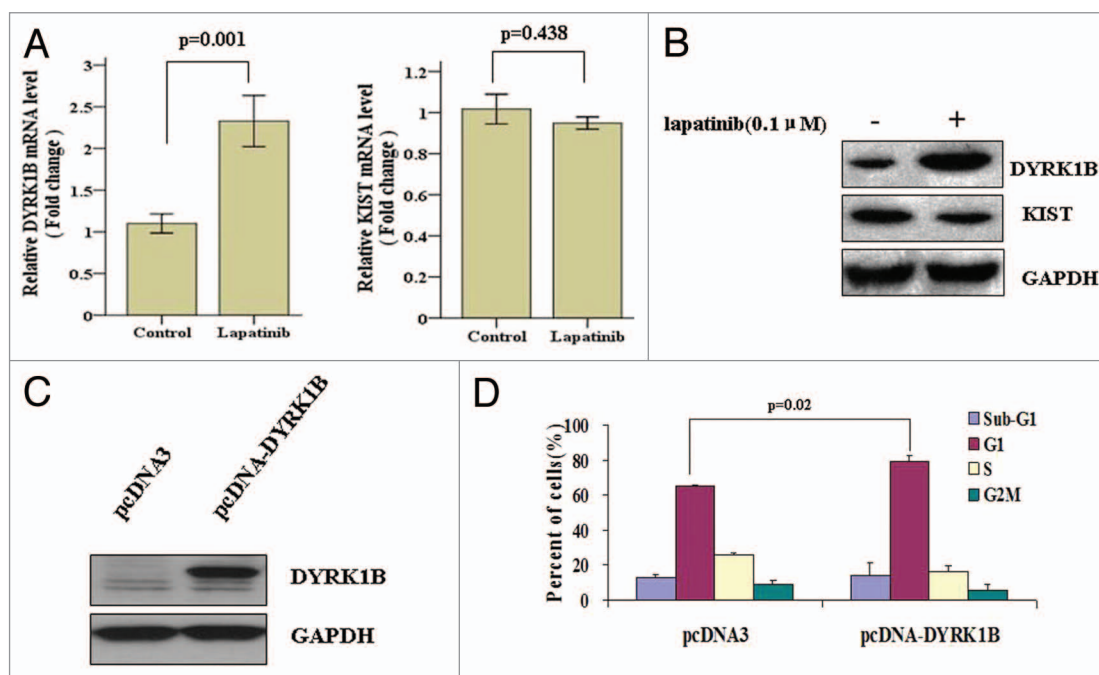


Figure 5. Lapatinib treatment increases DYRK1B expression in SK-BR-3 cells. (A) SK-BR-3 cells were treated with 0.1 μM lapatinib for 48 h, and mRNA expression levels of DYRK1B and KIST were determined by qRT-PCR. (B) SK-BR-3 cells were treated with 0.1 μM lapatinib for 48 h, and protein expression levels of DYRK1B and KIST were determined by western blot. (C) SK-BR-3 cells were transfected with a DYRK1B expressing plasmid or a vector-only plasmid, protein levels of DYRK1B were determined by western blot 48 h posttransfection. (D) SK-BR-3 cells were transfected with a DYRK1B expressing plasmid for 24 h, and then treated with 0.1 μM lapatinib for 48 h. Cell cycle distribution was quantified by flow cytometry analysis.

the stability of this protein. Ser10 phosphorylation is catalyzed by DYRK1B as well as KIST but is associated with different outcomes. p27 phosphorylation at Ser10 by KIST increases p27 binding through its nuclear export signal (NES) to CRM1 (or exportin 1), thereby promoting relocation of p27 from nucleus to cytoplasm, where it is degraded.^{50,51} In contrast, dual-specificity tyrosine-phosphorylation-regulated kinase 1B (DYRK1B, also called Mirk), a member of the conserved Dyrk/minibrain family of tyrosine regulated kinases, was shown to phosphorylate p27 Ser10 and stabilize p27 during the G₀ phase of the cell cycle by sequestering it within the nucleus, where it can bind to CDK2 and induce cell cycle arrest.⁴⁰ Although how cells can recognize and differentiate phosphorylation of the same site catalyzed by different kinases, leading to different outcomes, remains an interesting question, our data clearly show that lapatinib treatment increases DYRK1B, but not KIST expression. Thus, lapatinib inhibits nuclear export of p27 and stabilizes the protein in the nucleus, preventing its degradation.

Estrogen receptor (ER) β was discovered over a decade ago. Recent studies indicate that ER β has an anti-proliferative effect in breast cancer cell lines and in vivo.^{22,23,52} However, ER β has multiple isoforms, and their functions are not quite clear. We found that ER β 1 but not ER β 2 expression could upregulate p27 and enhance lapatinib-induced G₁ cell cycle arrest and cell proliferation inhibition in SK-BR-3 cells. These results suggest that ER expression status may affect lapatinib-induced p27 expression and cell cycle arrest. Therefore, more detailed analysis of ER status in breast cancer patients may be warranted, since expression of different ER β isoforms may predict different responses to lapatinib. Moreover, further functional studies of ER α and different ER β isoforms are needed, so that anti-estrogen therapies and other targeted therapies could be better formulated in a personalized manner.

Materials and Methods

Reagents

Lapatinib with a purity of 99% was purchased from Selleck Chemicals. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and cycloheximide (CHX) were from Sigma Chemicals. Cell Cycle Detection Kit was a product of Nanjing Kaiji Co. The antibodies used included the following: anti-p27 Ser10 (Abcam), anti-p27 Thr187 (Santa Cruz), anti-p27 (610241, BD Biosciences), anti-DYRK1B (AP7538b, Abgent), and anti-KIST (AP8067a, Abgent). All culture media and serum were obtained from GIBCO/BRL Life Technologies, Inc.

Cell lines and cell culture

SK-BR-3, MDA-MB-453, and MCF-7 cell lines were purchased from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin, and maintained at 37 °C with 5% CO₂ in a humidified atmosphere.

Cell viability

The effect of lapatinib on the viability of cells was determined by MTT assay. Human breast cancer cells were plated at a density of 3×10^4 cells per well in flat-bottomed 96-well plates. The next day, cells were provided with fresh medium including lapatinib at doses of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 μ M for another 24, 48, 72 h. After that the cells were incubated with 5 mg/L MTT solution at 37 °C for 4 h. The resulting crystal was dissolved in 200 μ l DMSO per well, and the plates were read on microplate reader at 570 nm. The effect of lapatinib on growth inhibition was assessed as percent cell viability, where DMSO-treated cells were taken as 100% viable. DMSO at the concentrations used

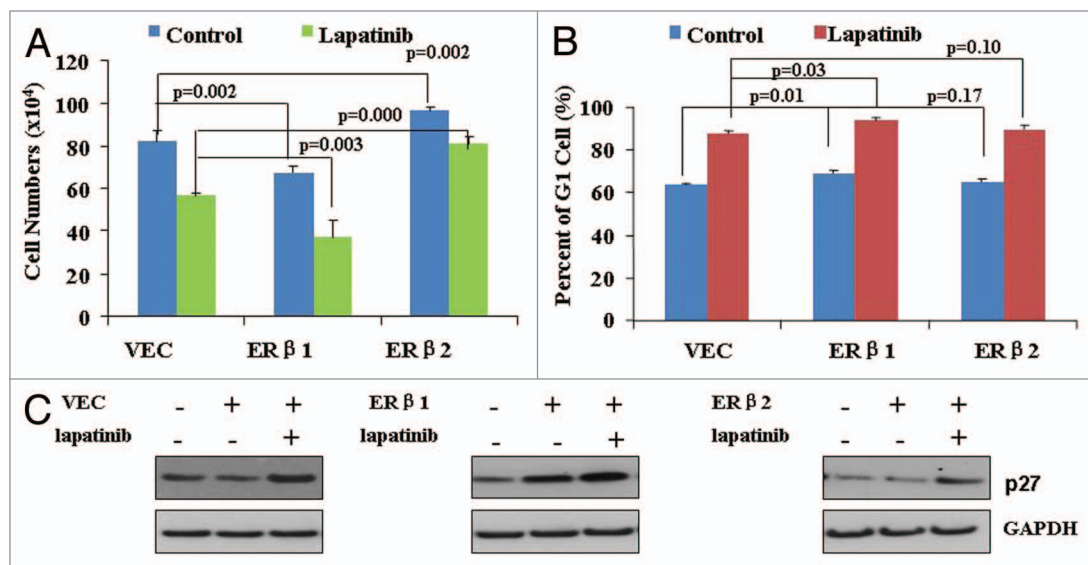


Figure 6. The effects of ER β 1 and ER β 2 on cell proliferation and G₁ cell phase arrest and p27 expression. (A) SK-BR-3 cells were transiently transfected with ER β 1- or ER β 2-expressing vectors, and treated 6 h later with 0.1 μ M lapatinib for 48 h. Cell proliferation was assayed by cell counting. (B) SK-BR-3 cells were transiently transfected with ER β 1- or ER β 2-expressing vectors and treated 6 h later with 0.1 μ M lapatinib for 48 h. Cell cycle distribution was quantified by flow cytometry analysis. (C) SK-BR-3 cells were transiently transfected with ER β 1- or ER β 2-expressing vectors, and treated 6 h later with 0.1 μ M lapatinib for 48 h. Protein levels of p27 were determined by western blot.

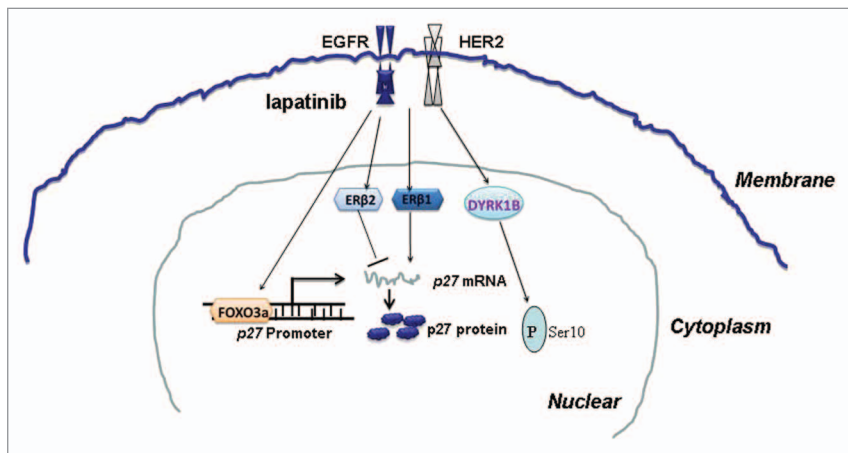


Figure 7. A schematic model for the roles of lapatinib in p27 expression regulation.

was without any effect on cell viability. Assays were performed 3 times independently.

Flow cytometry analysis

The cell cycle distribution was examined by flow cytometry. Briefly, the cells cultured with or without lapatinib, were trypsinized, washed with PBS, and fixed with 70% ethanol overnight at 4 °C. The fixed cells were washed with PBS and resuspended at the concentration of 1×10^6 cells/ml in a staining solution containing PI (20 $\mu\text{g/ml}$) and DNase-free RNase (100 $\mu\text{g/ml}$) and incubated for 30 min at 37 °C in the dark. Measurements and analyses were performed on a FACS Calibur flow cytometer with CELLQUEST software (BD Biosciences, San Diego). For each experiment, 30 000 events per sample were recorded.

RNA interference

p27-siRNA and nonspecific (control) siRNA were from Santa Cruz Biotechnology. Cells were seeded in 6-well culture plates at 2.5×10^5 cells per well (30–50% confluence) in RPMI-1640 medium supplemented with 10% fetal bovine serum. The next day, cells were transfected with p27 siRNA or a control siRNA at a final concentration of 100 nM by Lipofectamine2000 (Invitrogen). The next day, cells were fed with fresh medium with DMSO or lapatinib (0.1 μM) for 48 h, and harvested for cell cycle analysis or western blotting.

Transient transfections

SK-BR-3 cells at 60–70% confluency were seeded in a 12-well plate with 10% FBS. After 24 h, the cells were transiently transfected with Lipofectamine (Invitrogen, Inc) according to the manufacturer's protocol. Two hours before the transfection, the cells were fed with serum-free medium. Six hours after the transfection, the cells were fed with complete RPMI1640 medium, containing 10% FBS. Transient transfection of SK-BR-3 cells with ER β 1 or ER β 2 expression plasmid has previously been described.⁴¹

Cell proliferation assay

Three $\times 10^5$ SK-BR-3 cells were seeded in a 12-well plate with 10% FBS. After 24 h, the cells were transiently transfected with ER β 1- and ER β 2-expressing vectors or the control vectors. Six hours after the transfection, the cells were fed with complete

RPMI1640 medium containing 10% FBS. Forty-eight hours after transfection, the cells were counted with a Neubauer chamber for cell proliferation assay. In addition, cell proliferation was quantified by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay on a 96-well plate, as described in the manufacturer's protocol (Promega). The experiments were performed in triplicates and were repeated 3 times.

Western blot analysis

Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. The cell suspension was kept on ice for 20 min and then centrifuged at $15000 \times g$ for 15 min at 4 °C. Protein concentration was quantified with the BCA Protein Assay Kit. Protein samples were separated on a sodium dodecylsulfate-polyacrylamide gel and blotted onto nitrocellulose. Blots were blocked with 5% dry milk in tris-buffered saline/0.1% tween-20 and incubated overnight with a diluted solution of primary antibody, and then with the horseradish peroxidase-conjugated secondary antibody (1:50 000) for 2 h. The specific antibodies used were mouse anti-p27 (1:100 dilution), rabbit anti-phospho-p27 (1:1000 dilution), rabbit anti-MIRK/DYRK1B (1:100 dilution), rabbit anti-KIST (1:100 dilution), and mouse anti-GAPDH (1:5000 dilution). The blot was detected with the enhanced chemiluminescence detection system (Santa Cruz Biotechnology).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen) and was reverse transcribed using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. qRT-PCR was performed with SYBR[®]PrimeScript[®] RT-PCR Kit II. qPCR reactions for indicated genes were performed using SYBR green qPCR kit by a fluorescent temperature cycler (Mx3000P Real Time PCR System; Stratagene). Sequences of primers (forward and reverse, respectively) were as follows: p27: 5'-GTCAAACGTAACAGCTCGAAT-3' and 5'-TGCATAA TGCTACATCCAACG-3'; DYRK1B: 5'-GAGCTGATGAAC CAGCATGA-3' and 5'-CCTCAGGTGAGCGGTAGAAG-3'; KIST: 5'-CATTCTTTTGGCCCCTCATA-3' and 5'-GCTTTGGAATCACCAGCATT-3'; FOXO3a: 5'-AGGGAAGTTTGGTCAATCAGAA-3' and 5'-TGGAGATGAGGGAATCAAGTT-3'; GAPDH: 5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3'. Transcript quantification was done in triplicate for every sample and reported relative to GAPDH. The Relative gene expression was quantified according to the comparative Ct method using GAPDH as an internal standard.^{53,54}

Luciferase reporter assays

The reporter plasmids p27PF-Luc and control plasmid, pGVB2, were the generous gifts of Dr Toshiyuki Sakai (Kyoto Prefectural University of Medicine). SK-BR-3 cells (3×10^5 cells

per well) were seeded into 24-well plates and transfected the second day using Lipofectamine 2000 (Invitrogen). For transfection experiments, 0.8 μg of reporter plasmid, or pGVB2 plasmid and 0.2 μg of pCMV- β Gal expression plasmid were used. After 24 h of transfection, lapatinib, or DMSO (vehicle) were added and incubated for another 24 or 48 h. Cells were washed twice with cold PBS and lysed with 150 μl of cell culture lysis reagent (Promega). Thirty microliters of cell extract was used for luciferase activity assays, and 10 μl of cell extract was used for β -Gal assays. LumiCount was used to quantitate luciferase activity (Luciferase Assay System, Promega), and the β -Gal assay kit (Invitrogen, cat. #45-0449) was used for β -Gal activities following the kit protocol. All transfections were normalized by measuring β -galactosidase activity of the samples. The luciferase activity was normalized to β -Gal activity. Data are the average of at least 3 independent experiments, and error bars indicate standard deviation.

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Statistical analysis

The results were expressed as mean \pm SE calculated from the specified numbers of determination. A Student *t* test was used to compare individual data with control value. All analyses were performed at a significance level of $P < 0.05$ using SPSS version 16.0.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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