Original Article
Kinase inhibitors of HER2/AKT pathway induce ERK phosphorylation via a FOXO-dependent feedback loop

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Abstract: Inhibitors of the HER2/PI3K/AKT pathway are being developed, and shown promise in clinical trials for various types of cancers. However, development of drug resistance is a challenging problem for therapy. Elucidating various adaptive pathways leading to resistance or reduced sensitivity to drugs targeting the HER2/PI3K/AKT pathway may provide new insights into countering the resistance. Epidermal growth factor receptor (EGFR, aka HER1), which can dimerize with HER2, can activate a cascade consisting of Ras/RAF/MEK/ERK, promoting tumorigenesis. Lapatinib inhibits the kinase activity of both HER1 and HER2. In the current study, we found that repeated treatment of HER2+ breast cancer cells with HER1/2 inhibitor Lapatinib led to increased phosphorylation of RAF, MEK, and ERK, while suppressing HER1 phosphorylation and reduced the active form of Ras, indicating existence of factor(s) activating RAF/MEK/ERK by bypassing RAS activation. Notably, the Lapatinib treatment-induced phosphorylation of ERK was dependent on FOXO transcription factors, which are also activated by Lapatinib-mediated suppression of AKT. Moreover, the Lapatinib-induced phosphorylation of RAF and ERK is inhibited by a pan-PKC inhibitor. Furthermore, the Lapatinib induced increased ERK phosphorylation is correlated with increased stability of c-Myc, which is known to be stabilized by ERK-mediated phosphorylation. Together, these results suggest that chronic inhibition of the HER1/2 by Lapatinib triggers a feedback loop to activate RAF/MEK/ERK pathway, in a FOXO dependent but Ras-independent manner.

Keywords: HER2, ERK, breast cancer, Lapatinib

Introduction

Overexpression of receptor tyrosine kinase (RTK) human epidermal growth factor receptor 2 (HER2) is observed in 15-20% of breast cancer patients [1]. Targeted therapies with Transtuzumab and Lapatinib, monoclonal antibody inhibiting HER2 signaling and small molecule kinase inhibitor targetting HER1/2, respectively, are used to treat breast cancer patients with amplification or overexpression of the *HER2* gene. While Lapatinib, a dual inhibitor of EGFR (HER1) and HER2, remains useful for treatment of metastatic disease [2], it induces upregulation of several receptor tyrosine kinases (RTKs) including HER3, reducing the drug sensitivity. This mechanism is found to be common to various drugs that target the HER2/PI3K/AKT pathway. The upregulation of the RTKs occurs at transcriptional level by direct binding of FOXO transcription factors to the RTK promoters [3]. FOXOs are a family of transcriptional regulators, and they are phosphorylated by AKT, resulting in cytoplasmic location and degradation by ubiquitination-mediated proteolysis [4].

Inhibition of AKT phosphorylation leads to reduced FOXO phosphorylation and increased transport into the nucleus. The nuclear FOXOs bind to promoters of FOXO target genes in sequence specific manner, leading to activation or repression. FOXOs are generally considered as tumor suppressing factors because of their role in protecting cells from oxidative stress and promoting apoptosis and cell cycle arrest [5, 6]. On the other hand, they also play a critical role in cellular stress response [7]. Moreover, we previously found that FOXOs are recruited to the promoter of c-Myc on treatment with

Lapatinib in certain HER2+ breast cancer cell lines to increase c-Myc transcription [8]. Lapatinib also inhibits EGFR signaling and downstream effectors such as PI3K and AKT [9], however, the impact of prolonged treatment of HER2+ cells with Lapatinib on the downstream effector extracellular regulated kinases (ERK)/MAPK was not clear.

It has been reported previously that dual inhibitor of PI3K and mTOR, BEZ235, upregulates the level of phosphorylated ERK (pERK), likely through induction of HER3, increased dimerization of HER2/HER3, and activation of downstream Ras/RAF/MEK cascade [10]. Increased ERK phosphorylation (activation) plays a crucial role in stability of oncogenic c-Myc, as Sears et al. reported that Ras activated pathways regulate c-Myc protein stability via phosphorylation at serine 62 residue of ERK [11]. On the other hand, it is also known that inhibition of AKT leads to decreased phosphorylation of GSK3β at Serine 9, which phosphorylates c-Myc at threonine 58, resulting in ubiquitination and degradation of c-Myc [11]. In Burkitt's lymphoma, mutation of Thr-58, a major phosphorylation site in c-Myc and a mutational hot spot, increases c-Myc stability [12]. Thus ERK phosphorylation plays a crucial role in increasing protein stability of oncogenic c-Myc.

Our previous studies have shown that prolonged treatment of HER2+ breast cancer cells with Lapatinib gradually decreases sensitivity to the drug, despite downregulation of phosphorylation of AKT [8]. Since Lapatinib is a dual inhibitor of both HER2 and EGFR (HER1), which also activates Ras/RAF/MEK/ERK pathway, we further explored the impact of the prolonged treatment with Lapatinib on ERK signaling. We found that prolonged treatment of HER2+ breast cancer with Lapatinib or an AKT inhibitor leads to FOXO-dependent phosphorylation of ERK, independent of activation of Ras signaling, but through increased phosphorylation of RAF and MEK.

Materials and methods

Cell line and plasmids

BT474 cell line was a gift from Dr. Chodosh. MCF-HER2 cell line was a gift from Dr. Mien-Chie Hung at MD Anderson Cancer Center. BT474 and MCF-HER2 cells were cultured in DMEM containing 10% FBS and 1% Penicillin Streptomycin. Lapatinib was purchased from Selleck and AKTi-1/2/3 (MK-2206) was from Active Biochem; both drugs were dissolved in DMSO. FOXO inhibitor, AS1842856, was purchased from EMD Biosciences and dissolved in DMSO. FOXO1+3, IRS2 and INSR shRNAs were purchased from Open Biosystems. shRNA sequences can be found in the Supplemental Materials.

Western blot analysis

Cells were treated for indicated period of time, lysed in SDS buffer containing protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail (Roche). Protein supernatants (50-100 µg) were separated on 4-12% SDS gradient gels (geneScript), and transferred to nitrocellulose membranes. The primary antibodies used were Ras (Upstate), Raf-1 (SantaCruz Biotechnology), Phospho Raf-1 (Millipore), Mek, Phospho Mek, phospho Erk1/2, Erk1/2, GSK3β, GSK3β Phospho Serine 9, FOXO1 (Cell Signaling Technologies), FOXO3, c-Myc and FBW7 (Abcam). ECL detection system (Amersham Biosciences) was used for visualization of the bands.

Active Ras assay

BT474 cells were treated with DMSO or 200 nM Lapatinib daily for 4 days and collected 18 hours after the last treatment. The activity of Ras was determined using Ras activation assay kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, the cells were lysed in lysis buffer containing protease and phosphatase inhibitors, and the lysates were incubated with GST-Raf-1 RBD (Ras Binding Domain) at 4°C for 1 hour, followed by incubation with glutathione beads to pull down active Ras. GTPYS was used as a positive control while GDP as a negative control. Glutathione beads were washed with lysis buffer 4 times, the bound proteins were eluted in SDS sample buffer for Western blot analysis. The nitrocellulose membrane was stained with Ponceu S to determine Raf-1 RBD pulldown.

qRT-PCR

Total RNA was extracted from cultured cells using Trizol as previously reported [13]. RNA (1 µg) was used to make cDNA. Real time PCR
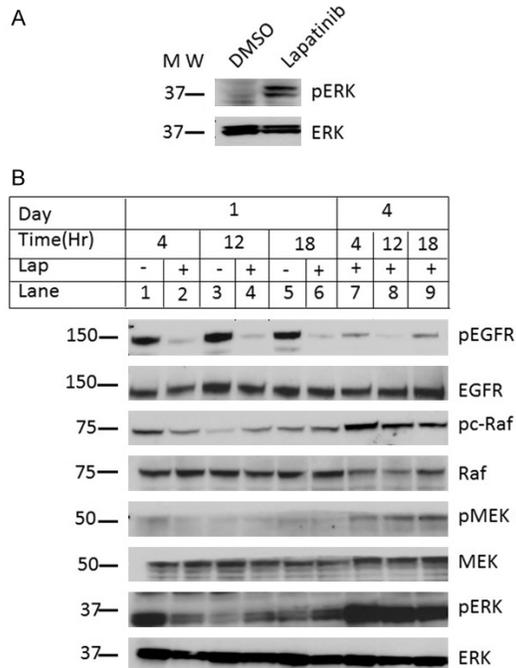
Figure 1. Lapatinib treatment decreases EGFR phosphorylation but increases RAF, MEK and ERK phosphorylation. A: BT474 cells were treated daily with DMSO or 200 nM Lapatinib for four days, collected 18 hrs after last treatment for Western blot. B: BT474 cells were treated with either DMSO or 200 nM Lapatinib for the indicated times on first day, or treated daily with Lapatinib for four days and collected after the indicated hr after the last treatment in the last day, for Western blot, with indicated antibodies.

was performed using 7500 fast real time PCR System. SDS software was used for data analysis. Sequence of the probes used can be found in Supplemental Information.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed according to the manufacturer's instructions (Imagenex Quick-CHIP kit, San Diego, CA). Approximately 5×10^6 cells were used for each immunoprecipitation. Briefly, cells were fixed with 1% formaldehyde and lysed in ChIP lysis buffer with protease inhibitors. The lysate was incubated with either control IgG or a specific primary antibody (4 μg) at 4°C overnight, and collected with protein G agarose beads. The protein-DNA complexes were eluted from the beads and incubated at 65°C overnight to reverse the protein-DNA crosslinking. DNA was amplified by PCR using primer pairs to detect indicated genes (Primer sequences are listed in the Supplemental Information) with SYBR Green reaction mix (Qia-

gen). The PCR product was quantified using an ABI fast 7500 real time quantitative PCR system. Reactions were done in duplicates and experiments were repeated twice and the mean normalized to input chromatin and reported as percent input.

Results

Lapatinib, a HER1/2 inhibitor, represses phosphorylation of EGFR, but increases phoasphorylation of ERK

Lapatinib is a dual inhibitor of HER2 and EGFR [2], and thus is also useful for inhibiting HER2/EGFR downstream kinases, PI3K/AKT and further downstream effectors such as mTOR. In most molecular and cellular studies about the impact of these inhibitors on cancers, the response of the cells to this inhibitor was evaluated after a single dose of or acute treatment with the inhibitors. In our previous study, we found that Lapatinib induces apoptosis of BT474 cells following the acute treatment, but prolonged daily treatment induces the adaptive response of the cells to the treatment by suppressing AKT, and activating FOXO/c-Myc axis [8]. We sought to determine the impact of repeated treatment with Lapatinib on the ERK pathway, which is separate from the PI3K/AKT pathway, but downstream of Ras/RAF effectors. To this end, we treated BT474 cells daily for 4 days, and found that phosphoERK (pERK) was upregulated in cells treated daily with Lapatinib, as compared to the DMSO treated cells (**Figure 1A**). Moreover, we also found that the upregulation of pERK required repeated treatments as cells treated with Lapatinib once for one day did not upregulate pERK (Figure S1, lanes 1-2 vs 3-4). The upregulation of pERK was not affected by the status of pAKT as Lapatinib treatment did downregulate the pAKT levels in cells treated only once or repeated for four days (Figure S1, lanes 2 and 4).

To further investigate the effect of daily Lapatinib treatment on ERK phosphorylation, in a time dependent manner, we treated BT474 cells for 4, 12 and 18 hours on day 1 (naïve cells) or treated daily for 4 continuous days, and collected the cells at 4, 12 or 18 hours following the last treatment, and then performed Western blot analysis. Our results show that Lapatinib treatment downregulated phospho-EGFR (pEGFR) on day 1 as well as day 4, as
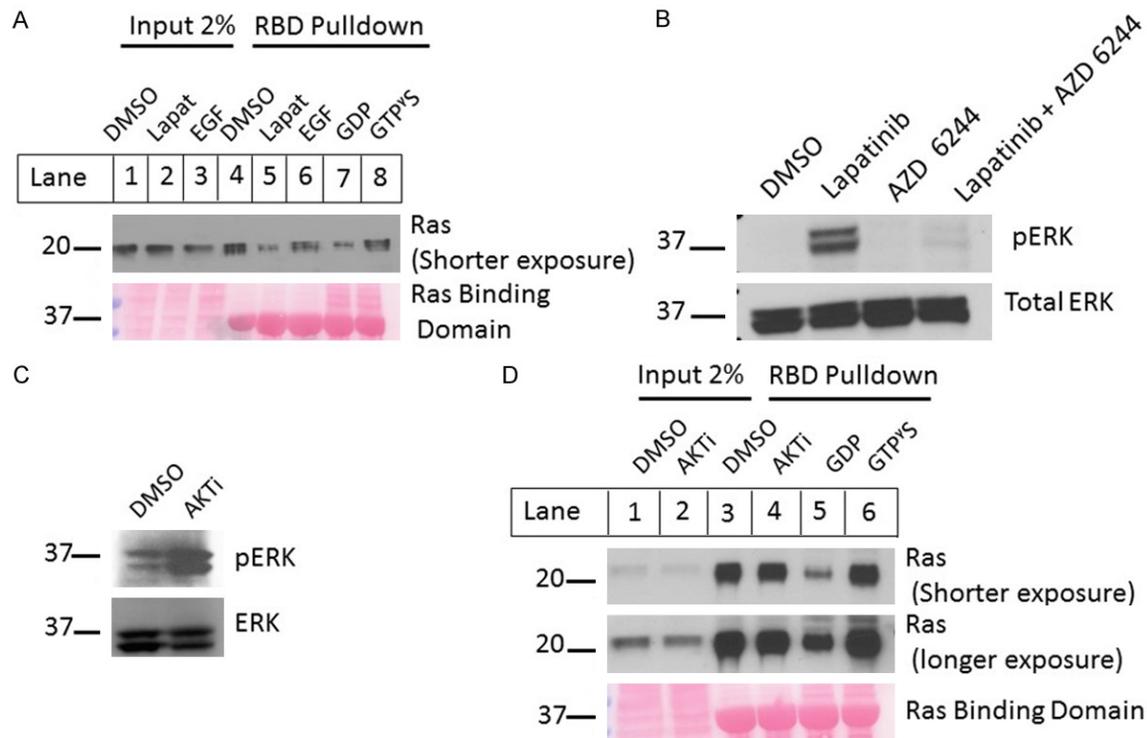
Figure 2. Lapatinib-induced ERK phosphorylation is independent of Ras activation. (A) BT474 cells were treated with either DMSO or Lapatinib daily for four days, collected 18 hrs after last treatment, or treated with 200 nM EGF for 30 min, and active Ras was assayed as described in materials and methods. (B) BT474 cells were treated with DMSO, 200 nM Lapatinib, 200 nM AZD6244 or a combination of 200 nM Lapatinib and 200 nM AZD6244 for 4 days, cells were collected 18 hours after last treatment and Western blot was performed (C) BT474 cells were treated with either DMSO or 200 nM MK2206 for four days, and collected 18 hrs after last treatment for Western blot. (D) The cells were treated with either DMSO or 200 nM MK2206 daily for four days, collected 18 hr after last treatment and active Ras assay was performed as described in (A).

expected (**Figure 1B**, lanes 2, 4, and 6-9). Consistent with downregulation of pEGFR by Lapatinib, in cells acutely treated with Lapatinib (within 1 day), the effectors of the EGFR downstream pathway, such as pRAF, pMEK, and pERK, were all inhibited after 4 hrs treatment, based on their reduced phosphorylation, as expected (**Figure 1B**, lanes 2 vs 1). However, in cells with changed medium containing either DMSO or DMSO/Lapatinib for 12 and 18 hrs, the levels of pRAF, pMEK, and pERK were all lower than that in the control lane 1 (**Figure 1B**, lanes 3-6 vs 1), likely due to time sensitive factors affected by change of medium in this window of time. Unexpectedly, after prolonged treatment (4 days), even though pEGFR remained inhibited (**Figure 1B**, lanes 7-9), its downstream effectors, such as pRAF, pMEK, and pERK were all upregulated based on their increased phosphorylation (**Figure 1B**, lanes 7-9). These findings indicate that acute treatment of the cancer cells with the

HER2/EGFR inhibitor suppresses EGFR and its downstream signaling. In contrast, a prolonged inhibition paradoxically activates its downstream effectors, suggesting existence of a feedback mechanism, leading to ultimate activation of pERK in the treated cells.

Lapatinib-induced phosphorylation of ERKs is independent of Ras activation

To determine how Lapatinib induces activation of the ERK pathway, considering the classic EGFR/RAS/RAF/ERK cascade, we explored whether Lapatinib induced activation of ERK partly by altering its key upstream regulator Ras. To do so, we treated BT474 cells daily for 4 days with control vehicle or Lapatinib, or EGF, in the presence of 10% of FBS, and collected cells 18 hrs after the last treatment to detect amount of active Ras (GTP-Ras), using an active Ras binding domain (RBD) for pulldown assay [14]. As a control, the total amount of Ras did

Lapatinib	-	+	-	+
shScram	+	+	-	-
shFOXO1+ 3	-	-	+	+
Lane	1	2	3	4
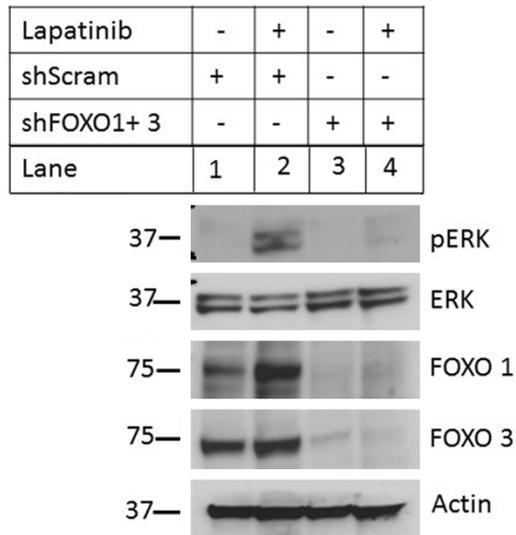
Figure 3. FOXO knockdown reduces Lapatinib-induced ERK phosphorylation. BT474 cells were transduced with either scramble or FOXO1 and 3 shRNAs, followed by treatment with either DMSO or Lapatinib for four days, collected 18 hr after the last treatment for Western blot.

not change following the treatment (**Figure 2A**, lanes 1-3, input). In contrast, treatment with Lapatinib substantially reduced the detection of active Ras (**Figure 2A**, lane 5 vs 4). Either EGF treatment or GTPγS did not increase the amount of active Ras (**Figure 2A**, lanes 6 and 8), suggesting that most Ras protein is in a highly activated state in the normal culturing and proliferative conditions. Collectively, these findings indicate that Lapatinib-induced phosphorylation of ERK likely resulted from Lapatinib-induced activation of c-RAF or MEK, but not the upstream Ras. Moreover, MEK inhibitor, AZD6244 blocked the upregulation of pERK on treatment with Lapatinib (**Figure 2B**), indicating increased phosphorylation of ERK is dependent on MEK.

Since phosphorylation of ERK was upregulated by chronic Lapatinib treatment independent of Ras signaling, we investigated whether the AKT pathway can play a role in upregulation of pERK. To this end, we treated BT474 cells with AKT inhibitor (AKTi), MK2206, daily for 4 days. Notably, treating the cells with AKTi also upregulated pERK (**Figure 2C**). Further, to understand whether activation of Ras plays any role in this activation, we performed active Ras binding assay with BT474 cells treated daily with either control vehicle or AKTi for 4 days. The results

showed that AKTi did not reduce the amount of active Ras in the cells (**Figure 2D**, lane 4), while as a control, GDP incubation substantially reduced the amount of GTP bound Ras (**Figure 2D**, lane 5). These results indicate that induction of pERK by either Lapatinib or AKTi is independent of upstream Ras activation.

Transcription factors FOXOs are crucial for Lapatinib-induced phosphorylation of ERKs

Both Lapatinib and AKTi can inhibit AKT, which normally suppresses transcription factors FOXOs by increasing their phosphorylation and sequestration in the cytosplasm, leading to their repression [15]. We explored whether Lapatinib and AKTi could both activate ERK by upregulating FOXOs. It is not known whether FOXOs play any role in regulating RAF, MEK and ERK independent of Ras pathway in the HER2+ cells. On the other hand, it has been suggested that increased ERK phosphorylation, after treatment with PI3K/mTOR inhibitor, BEZ235, results from increased expression of HER3 that dimerizes with HER2 [10]. Our results showed that increased phosphorylation of ERK following prolonged treatment with Lapatinib/AKTi is independent of active Ras. As such, we investigated whether FOXOs play a role in upregulation of this pathway. To this end, we knocked down FOXO1 and 3 with shRNA, and treated the resulting cells with DMSO or Lapatinib daily for 4 days. We found that knockdown of FOXO1 and 3 reduced the upregulation of pERK by Lapatinib treatment (**Figure 3**, lanes 3 and 4), suggesting that upregulation of pERK by Lapatinib is at least partly via regulating FOXOs.

Lapatinib induces expression of insulin receptor substrate 2 (IRS2), but IRS2 is not required for Lapatinib-induced ERK phosphorylation

Since FOXOs are crucial for induction of pERK by Lapatinib treatment, we determined whether FOXO target genes such as IRS-2 or Insulin Receptor (IR) play any role in pERK upregulation. First, we investigated whether daily treatment with Lapatinib affects IRS2 expression, based on Western blot analysis, and found that Lapatinib induced IRS2 expression (**Figure 4A**, lane 2). Similarly, treatment of MCF-HER2 cell line with AKTi also led to upregulation of IRS2 and pERK (Figure S2). To determine whether this upregulation is a FOXO-mediated process, we knocked down FOXO1 and 3 with cognate

A

Lapatinib	–	+	–	+
shScram	+	+	–	–
shFOXO1+3	–	–	+	+
Lane	1	2	3	4

B

C

Lapatinib	–	+	–	+	–	+
shScram	+	+	–	–	–	–
shIRS2	–	–	+	+	+	+
Lane	1	2	3	4	5	6
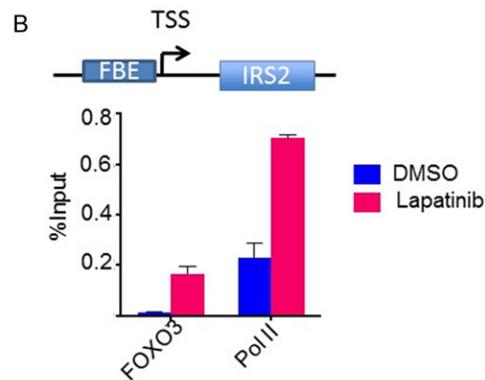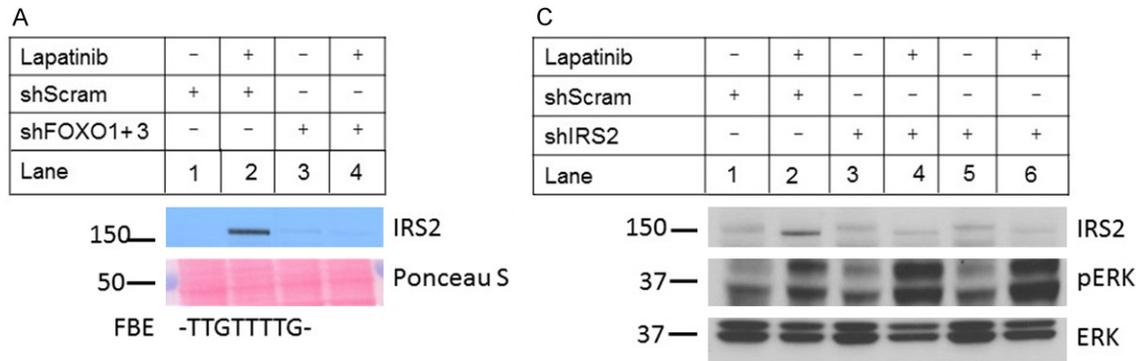
Figure 4. IRS2 knockdown does not affect Lapatinib-induced ERK phosphorylation. A: BT474 cells were transduced with either scramble or shRNAs targeting FOXO1 and 3, followed by treatment with Lapatinib daily for four days. The cells were collected 18 hr after the last treatment for Western blotting. B: BT474 cells were treated with DMSO or Lapatinib for three days, collcted 18 hrs after the last treatment for ChIP assay using primers for the IRS2 promoter region. C: BT474 cells were transduced with either scramble or shRNA targeting IRS2, and treated with either DMSO or lapatinib for four days, and collected 18 hrs after the last treatment for Western blotting with the indicated antibodies.
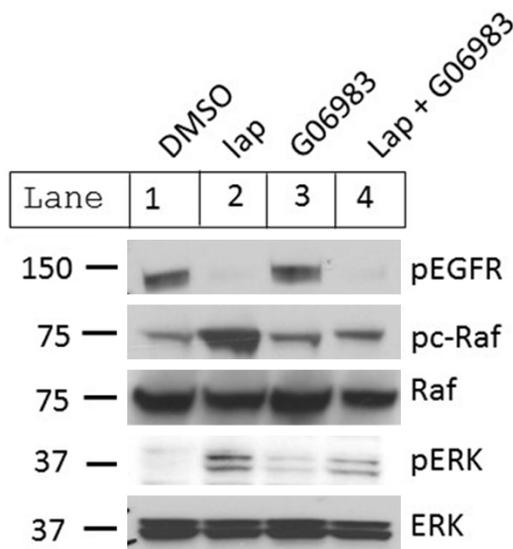
Figure 5. A pan-PKC inhibitor decreases Lapatinib-induced phosphorylation of RAF. BT474 cells were treated with either DMSO, Lapatinib, G06983 or combination of Lapatinib and G06983 daily for four days, and collected 18 hrs after the last treatment for Western blotting with the indicated antibody.

shRNAs, and treated the resulting cells with either DMSO or Lapatinib daily for 4 days. We found that FOXO knockdown reduced Lapatinib-in-

duced IRS2 expression (**Figure 4A**, lanes 4). Further, we tested whether FOXOs can directly upregulate the expression of IRS2 by directly binding to the IRS2 promoter using ChIP assay. Our results show that FOXO3a directly bound to the IRS2 promoter, and the binding was correlated with increased recruitment of Polymerase II to the promoter (**Figure 4B**).

To further investigate the role of IRS2 in pERK induction, we knocked down IRS2 and treated the resulting cells with DMSO or Lapatinib. IRS2 expression increased on treatment with Lapatinib (**Figure 4C**, lane 2). However, IRS2 knockdown did not affect phosphorylation of ERK induced by Lapatinib treatment (**Figure 4C**, lanes 2, 4 and 6). We also knocked down insulin receptor (IR) using shRNAs, and treated the resulting cells with either DMSO or Lapatinib, and our results show that IR knockdown also did not block the induction of pERK by Lapatinib (Figure S3).

A pan-active PKC inhibitor downregulates Lapatinib-induced ERK phosphorylation

Since PKC is known to activate phosphorylation of c-RAF independent of Ras signaling [16], we
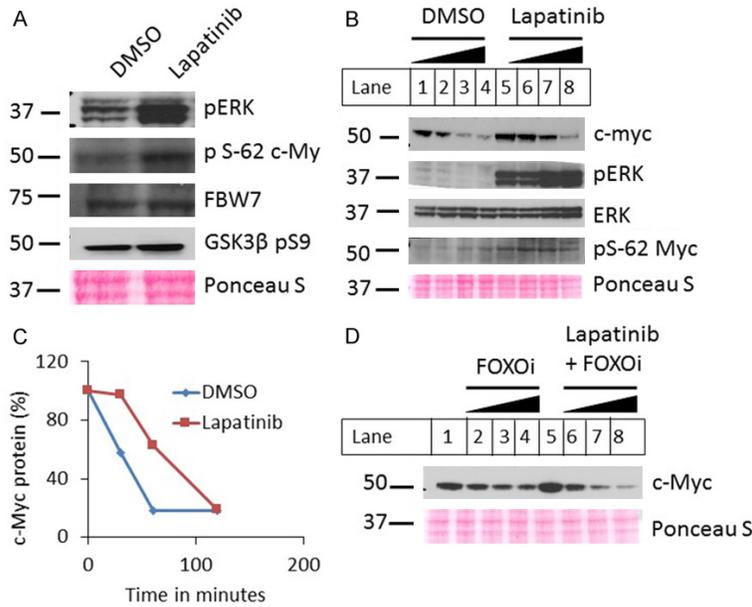
Figure 6. Lapatinib treatment increases stability of c-Myc protein. A: BT474 cells were treated daily with either DMSO or Lapatinib for 4 days and collected 18 hrs after the last treatment for Western blotting. B: BT474 cells were treated daily with either DMSO or Lapatinib for four days. Cycloheximide (20 μg/ml) was added and cells were collected at 0, 30, 60 and 90 min after treatment for Western blotting with the indicated antibodies. C: c-Myc protein was quantitated using Image J and plotted as % protein relative to 0 min. D: BT474 cells were treated with FOXOi without or with daily Lapatinib treatment for four days, followed by treatment with cycloheximide for 0, 30, 60 and 90 min. Cells were collected for Western blotting.

investigated whether a pan-PKC inhibitor inhibits Lapatinib-induced phosphorylation of ERK. To this end, we treated BT474 cells with either control vehicle, Lapatinib, PKC inhibitor (GO6-983), or a combination of Lapatinib and PKCi daily for 4 days. Treatment of the cells with the PKC inhibitor reduced induction of pRAF as well as pERK (**Figure 5**, lane 4). These results suggest that an isoform of PKC may be at least partly responsible for Lapatinib-induced activation of ERK.

Lapatinib treatment increases ERK phosphorylation, ERK-mediated phosphorylation of c-Myc, and c-Myc stability

The PI3K/AKT and ERK pathway plays a crucial role in c-Myc protein stability [11]. On one hand, pAKT promotes phosphorylation of GSK3β at serine 9, which leads to decreased phosphorylation of c-Myc at threonine 58, increasing stability of c-Myc [17]. Thus, blockade of pAKT by treatment with Lapatinib/AKTi could lead to increased degradation of c-Myc. On the other hand, increased pERK leads to phosphorylation of c-Myc at serine 62, leading to increased stability of the protein [18]. In our study, we found that although daily treatment of BT474 cells with Lapatinib/AKTi downregulated pAKT (Figure S1) [8], it did not downregulate GSK3β serine 9 phosphorylation (**Figure 6A**). At the same time, it also increased phosphorylation of ERK (**Figure 6A**). Thus, we hypothesized that prolonged treatment of BT474 cells with Lapatinib may increase stability of c-Myc protein via upregulation of pERK, leading to increased phosphorylation of c-Myc at serine 62. We found that Lapatinib treatment led to increased expression of pERK and phosphor S62 c-Myc, but did not affect expression of FBW7, a SCF ubiquitin ligase that degrades c-Myc [19] (**Figure 6A**, lane 2). Similarly, treatment of MCF-HER2 cells with AKTi increased the expression of phosphor S62 c-Myc (Figure S2). There was also slight increase in the expression of GSK3β phosphorylation at serine 9, despite downregulation of pAKT.

To test whether increased expression of phospho-S62 c-Myc is correlated with increased c-Myc protein stability, we treated BT474 cells daily with DMSO/Lapatinib for 4 days, followed by treatment with cycloheximide for 0, 30, 60 and 90 minutes. Western blot analysis showed that prolonged treatment with Lapatinib indeed increased c-Myc protein stability (**Figure 6B**, lanes 5-8 vs 1-4, and **Figure 6C**). As expected, the amount of pERK was increased after Lapatinib treatment (**Figure 6B**, lanes 5-8). We also observed increased level of pS62 of c-Myc (**Figure 6B**, lanes 5-8), indicating that increased stability of c-Myc likely resulted from increased phosphorylation at S62 of c-Myc by pERK induced by prolonged Lapatinib treatment.

Further, to investigate the role of FOXO in c-Myc protein stability, we treated BT474 cells with

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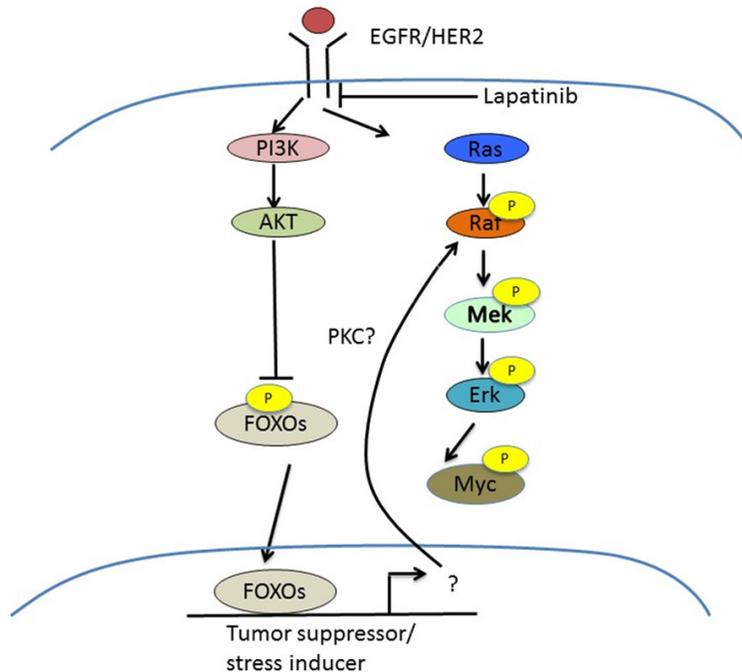
Figure 7. A working model for Lapatinib/AKTi-induced ERK phosphorylation and incresed c-Myc stability via a FOXO-mediated and a kinase dependent feedback loop.

enhanced transcription of oncogenic c-Myc in ER+/HER2+ breast cancer cell lines, resulting in reduced sensitivity of the cancer cells to HER1/HER2 inhibition [8]. Study of these compensatory adaptive pathways is important to design a mechanism-based and effective combination therapy.

Previously we found that although acute treatment of HER2+ breast cancer cells with Lapatinib induces apoptosis and repression of proliferation, the surviving cells soon adapt to the treatment by adopting a quiescent state with subdued proliferation, a state likely similar or equivalent to dormancy [8]. When the drug was removed for a longer period of time, the cells start to proliferate and are sensitive to drug treatment, indicating that the "dormant cells" can enter to the cycling phase given the time and relieve from drug-mediated suppression.

either DMSO or Lapatinib in presence of FOXO inhibitor, AS1842856, which prevents transactivation of FOXO target genes [20] for 4 days, and then with Cycloheximide for 0, 30, 60 and 120 minutes. Our results show that c-Myc protein degraded faster when treated with FOXO inhibitor, following Lapatinib treatment (**Figure 6D**, lanes 5-8), indicating the increased protein stability was FOXO-dependent.

Discussion

The HER1/2/PI3K/AKT pathway is deregulated in a number of cancers including breast cancer and drugs targeting this pathway are currently being developed at a rapid pace [21]. However, resistance to these drugs develops via compensatory or adaptive mechanisms [22]. AKTi, MK2206, upregulates expression of various receptor tyrosine kinases via FOXO-induced gene transcription, leading to resistance to PI3K/AKT inhibitors [3, 23]. Moreover, it has been reported that inhibition of the HER2 signaling by PI3K inhibitor in breast cancer cells leads to activation of a compensatory pathway, the RTK/Ras-activated RAF/MEK/ERK pathway, to protect the cancer cells [10]. We recently show that daily treatment with Lapatinib leads to

It has been reported that short term treatment of HER2+ breast cancer cells with PI3K inhibitor suppresses PI3K/AKT pathway, a proproliferative pathway, but activates RAS/ERK pathway due to increased expression of HER2/HER3 signaling that activates the RAS/ERK pathway [10]. Our prolonged treatment of the HER2+ breast cancer cells with Lapatinib also led to upregulation of the RAF/MEK/ERK pathway. However, unlike the previous setting, the prolonged treatment of the cancer cells with Lapatinib, or similarly with AKTi, activates ERK not through the RTK/Ras pathway, as we did observe that these inhibitors reduced phosphorylation of EGFR and inhibited Ras. These findings unravel a novel and time-dependent but Ras-independent pathway that activates ERK phosphorylation, following the prolong inhibition with either Lapatinib or AKTi (**Figure 7**).

We previously found that FOXOs can directly bind to c-Myc promoter to increase its transcription in HER2+ cell lines [8]. In the current study we showed that Lapatinib-induced phos-

phorylation of ERK requires the participation of FOXOs as knockdown both of FOXO1 and FOXO3 reduced Lapatinib-induced ERK phosphorylation.

Our findings indicate that chronic treatment of the HER2+ cells with Lapatinib induced phosphorylation of RAF/MEK/ERK pathway, but did not affect the upstream Ras, a key node leading to the activation of RAF/MEK/ERK, Instead, the Lapatinib-induced ERK phosphorylation was attenuated by treatment with a pan-PKC inhibitor. These findings suggest a possible protein kinase(s) activated by the Lapatinib treatment and this kinase may lead to phosphorylation and activation of RAF, bypassing the upstream Ras, resulting in phosphorylation of ERK (**Figure 7**). As there are many kinases in this family, coupled with the possibility some members have redundant functions, we are currently unable to identify the responsible kinases.

In summary, our findings suggest a working model where by prolonged treatment of the HER2+ caner cells leads to activation of FOXOs which can bind the promoter of various genes likely including a PKC related protein kinases (**Figure 7**). Our current studies uncover a distinct pathway beyond transcription regulation, to increase the stability of c-Myc. Moreover, our findings also unravel a new Lapatinib-induced feedback pathway to activate ERK in a Ras independent manner, via a Lapatinib-mediated, FOXO-dependent factor(s) to activate RAF/MEK/ERK pathway (**Figure 7**). This feedback loop is distinct from the PI3K/AKT-induced activation of RTK/RAS/RAF pathway. It is likely that the Lapatinib/PI3K/AKT inhibitors induce a series of time sensitive response and feedback loops to trigger the resistance/adaptive response, and our findings provide additional mechanisms/targets to be tested for improving the therapy of HER2+ cancer.

Acknowledgements

Disclosure of conflict of interest

None.

Authors' contribution

S. M. and X.H. designed the experiments and wrote the manuscript. S.M. performed majority of the experiments and CY.A performed some experiments.

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Supplemental materials and methods

Nucleotide suequence for shRNAs

Gene	shRNA sequence
FOXO1+3	TTATCCTTGAAGTAGGGCACG
IRS2	1. AAGTCGATGTTGATGTACTCG
	2. ATGTCTCCGTAGTCCTCTGGG
INSR	1. GTCTGTTACTTGGCCACTAT
	2. CACTGATTACTTGCTGCTCTT

Nucleotide sequence for qRT-PCR

INSR Forward: GTACCGCGAAGGGCAAGA
Reverse: CACTCGTGGCAACAGAG

Nucleotide sequence for ChIP assay

IRS2 promoter
Forward: CTGTGTGTGCCTGCGTAAC
Reverse: CTGCTGCTGGTGTTGCTGC
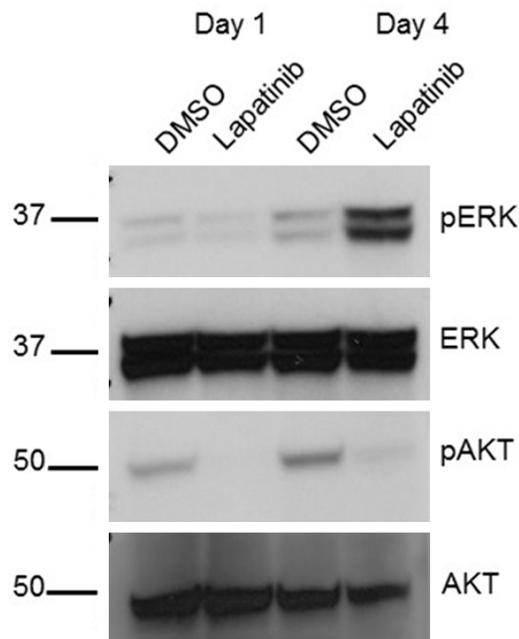
Figure S1. Repeated treatment of BT474 cells with Lapatinib increases the expression of pERK despite downregulation of pAKT. BT474 cells were treated with DMSO or 200 nM Lapatinib for 18 hrs or daily for 4 days and collected 18 hrs after the last treatment for Western blotting with indicated antibodies.
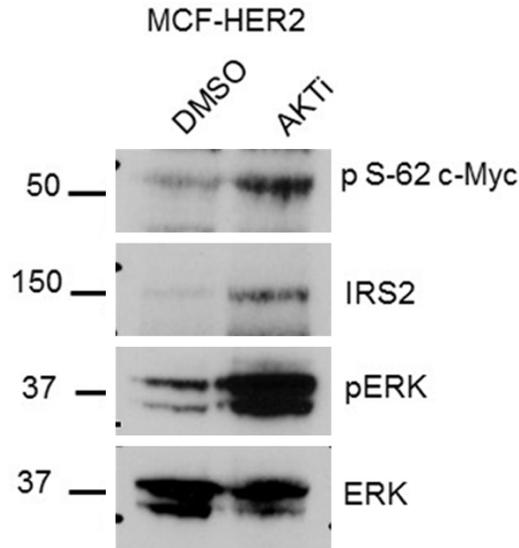
MCF-HER2

Figure S2. *AKT inhibitor induces ERK phosphorylation. MCF-HER2 cells were treated with either DMSO or MK2206 daily for four days, collected 18 hrs after the last treatment for Western blotting using the indicated antibodies.*

A

B

Lapatinib	–	+	–	+	–	+
shScram	+	+	–	–	–	–
Sh INSR	–	–	+	+	+	+
Lane	1	2	3	4	5	6
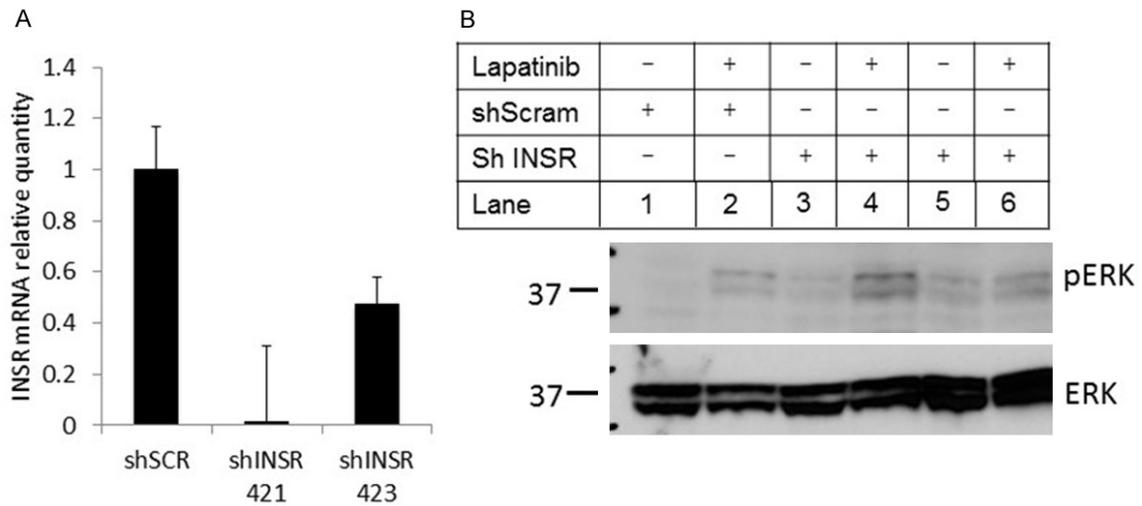
Figure S3. A: BT474 cells were transduced with either scramble or shRNA targeting INSR, and collected for RNA preparation and real time RT-PCR analysis for INSR mRNA level. B: BT474 cells were transduced with either scramble or shRNA targeting INSR, and the resulting cells were treated with either DMSO or Lapatinib for four days, and collected 18 hrs after the last treatment and Western blotting.