이학박사 학위논문

EGFR 저해제의 작용 및 내성기전 연구

Identification of predictive biomarkers and the mechanisms of acquired resistance to epidermal growth factor receptor inhibitors

2014년 2월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

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연구

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이 논문을 이학박사 학위논문으로 제출함

2013년 10월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

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김 황 필의 이학박사 학위논문을 인준함

2013년 12월

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Identification of predictive biomarkers and the mechanisms of acquired resistance to epidermal growth factor receptor inhibitors

by

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A Thesis Submitted to the Interdisciplinary Graduated Program in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Cancer Biology at the Seoul National University College of Medicine

December 2013

Approved by thesis committee:

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ABSTRACT

Identification of predictive biomarkers and the mechanisms of acquired resistance to epidermal growth factor receptor inhibitors

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Major in Cancer Biology

Interdisciplinary Graduate Program

The Graduated School

Seoul National University

Epidermal growth factor receptor (EGFR) is one of the most commonly altered genes in human cancer by way of over-expression, amplification, and mutation. Targeted inhibition of EGFR activity suppresses signal transduction

pathways which control tumor cell growth, proliferation, and resistance to apoptosis. EGFR targeted therapies have been recently developed for the treatment of multiple cancer types. At the time when they were introduced in clinical practice, there was little knowledge of the molecular bases of tumor sensitivity and resistance to these novel targeted compounds.

First, epidermal growth factor receptor tyrosine kinase inhibitor has been shown to exert a synergistic antitumor effect when combined with fluoropyrimidine. This synergy may be attributable to the downregulation of thymidylate synthase (TS), which is frequently overexpressed in fluoropyrimidine-resistant cancer cells. However, the molecular mechanism underlying the downregulation of TS has yet to be clearly elucidated. I have demonstrated that lapatinib, a dual inhibitor of EGFR and HER2 TK, effectively downregulates a variety of nucleotide synthesis-related genes, including TS, and exhibits activity superior to that of gefitinib not only in HER2-amplified cells, but also in wild-type cells. As a mechanism, I have determined, for the first time, that nuclear EGFR and HER2 activate TS gene transcription, and that EGFR and HER2-bound TS promoter activities are inhibited by lapatinib treatment.

Second, although non-small cell lung cancer (NSCLC) cells with somatic

mutations in EGFR initially show a dramatic response to tyrosine kinase inhibitor, these cells eventually develop resistance to TKI. This resistance may be caused by a secondary T790M mutation in the EGFR tyrosine kinase, which leads to the substitution of methionine for threonine in 790. I showed that lapatinib revealed moderately enhanced cytotoxicity against gefitinib-resistant T790M cells in vitro and in vivo. Based on its dedimerization effect on EGFR and HER2, lapatinib may partially attenuate the resistance to EGFR TKI in T790M lung cancer cells.

Third, human epidermal growth factor receptor 2 (HER2)-directed treatment using trastuzumab has shown clinical benefit in HER2-positive gastric cancer. Clinical trials using lapatinib in HER2-positive gastric cancer are also currently underway. As with other molecularly targeted agents, the emergence of acquired resistance to HER2-directed treatment is an imminent therapeutic problem for HER2-positive gastric cancer. The measurement of basal gene expression levels allowed us to identify Testican-1-mediated EMT as a mechanism of acquired resistance to lapatinib in HER2-gastric cancer cells. Testican-1 silencing partially restored sensitivity to lapatinib in resistant cells, implying that other signaling pathways may also be responsible for drug resistance. However, owing to the limited number of cell lines used in our

experiments, it is possible that other important biological factors exist that contribute to drug resistance in HER2-positive gastric cancer.

Key words: EGFR, HER2, gefitinib, lapatinib, cetuximab, gefitinib resistance, Testican-1, epithelial-mesenchymal transition, lapatinib resistance

Student number : 2002-23756

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I. Background and Purpose

1. Oncogene addiction

Cancer cells contain multiple genetic and epigenetic abnormalities. Despite this complexity, their growth and survival can often be impaired by the inactivation of a single oncogene. This phenomenon, called “oncogene addiction,” provides a rationale for molecular targeted therapy [1, 2] . The efficacy of this strategy requires novel methods, including integrative genomics and systems biology, to identify the state of oncogene addiction (i.e., the “Achilles heel”) in specific cancers. Combination therapy may also be required to prevent the escape of cancers from a given state of oncogene addiction.

The most convincing evidence for the concept of oncogene addiction comes from the increasing number of examples of the therapeutic efficacy of antibodies or drugs that target specific oncogenes in human cancers. The earliest example is the antibody trastuzumab (Herceptin), which targets the receptor tyrosine kinase HER-2/NEU in patients with breast cancer. More recent evidence is provided by the therapeutic efficacy of drugs that target various oncogenic protein kinases. Examples include imatinib, which targets the bcr-abl oncogene in chronic myeloid leukemia and also targets the c-kit oncogene in gastrointestinal stromal tumors, and gefitinib and erlotinib, which

target the epidermal growth factor receptor (EGFR) in non–small cell lung carcinoma (NSCLC), pancreatic cancer, and glioblastoma. It is of interest that the clinical responses in NSCLC are mainly confined to the subset of cancers that have mutations or amplification in the EGFR gene.

2. Epidermal growth factor receptor as targets for cancer therapy

The epidermal growth factor receptor is a member of the type 1 receptor tyrosine kinase family known as the ERBB family. Comprising 4 members-ERBB1, ERBB2 (also known as HER2), ERBB3 (HER3), and ERBB4 (HER4)-these receptors play a principal role in allowing cells to integrate and respond correctly to diverse external stimuli, ranging from soluble endocrine and paracrine factors to signaling molecules on neighboring cells. The cell must interpret these extracellular signals to produce an appropriate developmental or proliferative response, and aberrant activation of the kinase activity of these receptors, particularly EGFR and HER2, is important in the development and progression of human cancer [3]. Given its roles in signal transduction and development of the malignant phenotype, EGFR has

emerged as a critical target for therapeutic development against various forms of cancer [4]. Two different types of EGFR-targeted therapeutic agents were subsequently developed: mAbs, such as cetuximab and panitumumab, which target the extracellular domain of the receptor, thereby inhibiting ligand-dependent EGFR signal transduction; and small-molecule tyrosine kinase inhibitors, such as gefitinib and erlotinib, which target the intracellular tyrosine kinase domain of the EGFR. Furthermore, recent clinical and laboratory studies have identified molecular markers that have the potential to improve the clinical effectiveness of EGFR-targeted therapies

3. Acquired resistance to targeted therapy

Drugs that target genomically defined vulnerabilities in human tumors have now been clinically validated as effective cancer therapies. However, the relatively rapid acquisition of resistance to such treatments that is observed in virtually all cases significantly limits their utility and remains a substantial challenge to the clinical management of advanced cancers. As molecular mechanisms of resistance have begun to be elucidated, new strategies to overcome or prevent the development of resistance have begun to emerge. In

some cases, specific mutational mechanisms contribute directly to acquired drug resistance, and in other cases it appears that nonmutational and possibly epigenetic mechanisms play a significant role.

4. Purpose

Based on the background described above,

1) To identify the target and the molecular mechanism for synergistic effect of combination treatment with lapatinib and fluoropyrimidine for HER2-positive gastric cancer

2) To identify the molecular mechanism of acquired resistance to gefitinib in EGFR-mutant NSCLC

3) To understand the molecular mechanism of acquired resistance to lapatinib in HER2-positive gastric cancer

Ⅱ. Lapatinib downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2

ABSTRACT

Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) has been shown to exert a synergistic antitumor effect when combined with fluoropyrimidine. This synergy may be attributable to the downregulation of thymidylate synthase (TS), which is frequently overexpressed in fluoropyrimidine-resistant cancer cells. However, the molecular mechanism underlying the downregulation of TS has yet to be clearly elucidated.In this study, we demonstrate that lapatinib, a dual TKI of EGFR and HER2 downregulates TS via inhibition of the nuclear translocation of EGFR and HER2. From our cDNA microarray experiments, we determined that a variety of nucleotide synthesis-related genes, including TS, were downregulated with lapatinib, and this was apparent in HER2-amplified cells. Targeted and pharmacologic inhibition assays confirmed that the dual inhibition of EGFR and HER2 is required for the more effective reduction of TS as compared to what was observed with gefitinib or trasutuzumab alone. Additionally, I determined that co-transfected EGFR and HER2 activate the TS gene promoter more profoundly than do either EGFR or HER2 alone. The translocation of EGFR and HER2 into the nucleus and the subsequent

activation of the TS promoter were inhibited by lapatinib. These results demonstrate that lapatinib inhibits the nuclear translocation of EGFR and HER2 and downregulates TS, thus sensitizing cancer cells to fluoropyrimidine

INTRODUCTION

Lapatinib (GW572016, Tykerb) is a dual synthetic reversible inhibitor of EGFR and HER2 tyrosine kinases, and has been demonstrated to inhibit significantly the proliferation of cancer cells evidencing EGFR and/or HER2 overexpression both in vitro and in vivo [5-7]. At the intracellular level, lapatinib binds reversibly to the cytoplasmic ATP-binding site of the kinase, thereby preventing receptor phosphorylation [8]. Lapatinib blocks ligand-activated signaling from multiple receptor combinations, including homo-and heterodimers of EGFR and HER2 [9]; preclinically, it inhibits the proliferation of trastuaumab-resistant cancer cells [9]. Moreover, in contrast to trastuzumab, lapatinib can inhibit HER2 activation via ligand-induced heterodimerization or truncated HER2 receptors, and it has also proven effective in the treatment of PTEN-deficient breast cancer, thus illustrating the potential advantages of lapatinib over trastuzumab [10, 11].

Recently, lapatinib has been shown to exert beneficial effects in combination with capecitabine in patients with HER2-positive advanced breast cancer that has progressed after prior treatment with an anthracycline,

a taxane, and trastuzumab [12]. In this trial, the time to progression of patients treated with lapatinib and capecitabine was prolonged significantly as compared to what was observed in patients treated solely with capecitabine (8.4 months vs. 4.4 months, $p<0.001$), which suggests that lapatinib may overcome trastuzumab resistance. However, another possible explanation for this observed synergistic effect would be that lapatinib may enhance sensitivity to capecitabine. In this regard, several lines of inquiry have demonstrated that EGFR-TKIs inhibited the expression of the transcription factor E2F-1, thereby inducing the downregulation of TS expression and activity, and mediating the synergistic interaction with 5-FU [13, 14]. However, the molecular mechanism underlying the downregulation of TS remains to be clearly elucidated. Fluoropyrimidines such as 5-FU are extensively utilized in the treatment of colorectal, breast, and aerodigestive tract cancers, and are intracellularly converted to 5-fluoro- deoxyuridine-monophosphate (FdUMP), thus forming a stable tertiary complex and inhibiting TS [15-17]. The results of several studies have demonstrated that the expression of TS functions as a key determinant of fluorpyrimidine sensitivity, and preclinical in vitro and in vivo studies have elucidated an inverse relationship between TS expression in cancer cells and fluoropyrimidine sensitivity [18-20]. Thus, EGFR TKI may

represent a novel therapeutic strategy which can attenuate TS expression in cancer cells.

EGFR and HER2 are cell surface receptors which transduce mitogenic signals within the cells [4, 21]. However, the nuclear importation of EGFR and HER2 has been also demonstrated, although its biological significance remains unclear. EGFR has been detected in the nuclei of cancer cells and in primary tumor specimens of various origins, as well as in those of other highly proliferative tissues. While localized in the nucleus, EGFR may operate as a transcriptional regulator. It has been previously reported that nuclear EGFR regulates the expression of cyclin D1, inducible nitric oxide synthase (iNOS), and B-MYB genes via transactivational activity [22-24]. Furthermore, nuclear EGFR has been demonstrated to interact physically with signal transducer and activator of transcription 3 (Stat3) and E2F-1. Aside from EGFR, other receptors in the EGFR family, including HER2, have also been detected within the nucleus [25, 26], but the biological significance of these receptors will require additional study.

In this study, I attempted to determine the manner in which lapatinib renders cancer cells susceptible to fluoropyrimidine. I determined that EGFR and HER2 existed within the nucleus, and that nuclear EGFR and HER2 bind

to and activate the TS gene promoter. I further noted that lapatinib inhibits the nuclear translocation of EGFR and HER2, thereby induing a reduced association with the TS promoter. The lapatinib-mediated downregulation of TS was apparent in HER2-amplified cells; however, it was also noticeable in the wild-type cells. It is also important to note that the dual inhibition of EGFR and HER2 is the most effective method for achieving maximal TS downregulation. Taken together, these data show that lapatinib, a dual inhibitor of EGFR and HER2 TS, may prove useful not only as a targeted therapy, but also as a chemosensitizer of cytotoxic anticancer drugs in a specific subset of tumors.

MATERIALS AND METHODS

1. Reagents

Gefitinib was kindly provided by AstraZeneca, and lapatinib was generously provided by GlaxoSmithKline. Trastuzumab was kindly provided by Roche. 5-FU was obtained from Choong Woe (Seoul, Korea). Epidermal growth factor (EGF) was purchased from Sigma-Aldrich (St. Louis, MO).

2. Cell Culture

Four human gastric cancer cells (SNU216, SNU484, SNU668, N87), human breast cancer cells (SKBr3), and Chinese hamster ovary cells were grown at 37°Cunder 5% CO2 in RPMI-1640 or DMED culture media containing 10% fetal bovine serum (WELGENE Inc., Korea). The mycoplasma free cells were purchased from the Korean Cell Line Bank (Seoul, Korea, ref[27])or the American Type Culture Collection.

3. N87 Xenografts

All animal experiments were approved by the Institutie Laboratory Animal

Resources Seoul National University and Use Committee. Six-to-eight-week-old female BALB/c athymic (nu+/nu+) mice were purchased from Central Lab Animal Inc. (Seoul, Korea). The initial body weight of the animals at the time of arrival was between 18 and 20 g. Mice were allowed to acclimatize to local conditions for 1 week before being injected with cancer cells. Tumors were induced by injecting H1975 cells (5×106) subcutaneously into the right flank of mice. The tumors were then measured twice a week using calipers, and the tumor volume in mm3 was calculated according to following formula: {(width)2×(height))/2}. When tumors had reached a volume of 50–100 mm3, treatment with either lapatinib, 5-FU, a combination of lapatinib and 5-FU, or a vehicle control was initiated. Lapatinib were administered via oral gavage at a concentration of 100 mg/kg in 0.5% (W/W) hydroxypropylmethylcellulose (HPMC) with 0.1% (W/W) Tween80 (Sigma) in sterile milli-Q water Monday through Friday for 3 weeks. A dose of 50 mg/kg of 5-FU was given intraperitoneally once weekly for 3 weeks. Statistical analysis to compare tumor sizes in xenograft-bearing mice was performed with ANOVA. Differences between groups were considered statistically significant if $P<0.05$.

4. cDNA Microarray

SNU216, SNU484, and SNU668 cells were grown for 24 hours in the

presence of gefitinib (1 μmol/L) or lapatinib (1 μmol/L), after which they were lysed. The total RNA was then processed and hybridized to an Affymetrix Genechip HG-U133 set (Affymetrix, Santa Clara, CA) via a DNA link (Seoul, Korea) according to the manufacturer's protocols. All samples were analyzed and reported according to MIAME guidelines. The GeneExpress Software System Fold Change Analysis tool was used to identify all present genes expressed at least 2-fold greater in the drug-treated cells compared with DMSO-treated cells. For each gene fragment, the ratio of the geometric means of the expression intensities in DMSO treated cells and the drug treated cells cells was calculated, and the fold change was then calculated on a per fragment basis. Confidence limits were calculated using a two-sided Welch modified t test on the difference of the means of the logs of the intensities.

5. Reverse Transcription-PCR and Real-Time PCR Analysis

Specific mRNAs were semiquantitated via reverse transcription (RT-PCR) or real-time PCR with the iCycler IQ detection system (Bio-Rad Laboratories, Hercules, CA) using SYBR green I (Molecular Probe, Eugene, OR) in

triplicate reactions. The primers used in the PCR reaction were as follow: E2F1, forward primer5′- ACGCTATGAAACCTCACTAAA-3′ and reverse primer5′-AGGACATTGGTGATGTCATA-3′, TS, forward primer 5′-TCTGGAAGGGTGTTTTGGA-3′ and reverse primer 5′-CCTCCACTGGAAGCCATAAA-3′, TK1, forward primer 5′-CAGCTTCTGCACACATGAC-3′ and reverse primer 5′-AGTGCAGCCACAATTACGG-3′, DHFR, forward primer 5′-TCCATTCCTGAGAAGAATCGACCTT-3′ and reverse primer 5′-CACAAATAGTTTAAGATGGCCTGGG-3′, RRM2, forward primer 5′-GTGGAGCGATTTAGCCAAGA-3′ and reverse primer 5′-TGACCTCTTTGTCCCCAATC-3′, DUT, forward primer 5′-CCCTTCTGGGTGTTATGGGAAGA-3′ and reverse primer 5′-CCAGCTCCTACATCAATAAAGTGTTT-3′, NME1, forward primer 5′-TTCACCCTGAGGAACTGGTAGATT-3′ and reverse primer 5′-GTGGTCTGCCCTCCTGTCA-3′, ACTIN, forward primer 5′-AGAGCTACGAGCTGCCTGAC and reverse primer 5′-GGATGCCACAGGACTCCA-3′.

6. Antibodies and Western Blotting

Antibodies against E2F-1, α-tubulin, and Lamin B were purchased from Santa

Cruz Biotechnology (Santa Cruz, CA). p-EGFR (pY1068), EGFR, p-HER2 (pY1221/1222), HER2, and TS antibody was acquired from Cell Signaling Technology (Beverley, MA), or NeoMarkers (Fremont, CA). Cultured cells that had reached ~70% to 80% confluence were used for protein analyses. The cells were treatmented with different conditions as described. The cells were lysed in RIPA buffer on ice for 15 min (50 mmol/L Tris-HCl pH 7.5, 1% NP-40, 0.1% Na deoxycholate, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 1 mmol/L nitrophenolphosphate, 1 mmol/L benzamidine, 0.1 mmol/L PMSF, 0.1 mmol/L aprotinin, 0.1 mmol/L leupeptin, 0.1 mmol/L pepstatin A) and centrifuged at 13,000 rpm for 20 min. Samples containing equal amount of total protein were resolved in SDS-polyacrylamide denaturing gel, transferred to nitrocellulose membranes, and probed with antibodies. Detection was performed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

7. Cell Cycle Analysis

Cells were washed twice in phosphate buffered solution (PBS), fixed in 70% ethanol, and stored at −20°C until required for analysis. Before analysis, cell

suspensions were washed with PBS, and digested with RNase A (50 μg/ml) for 15 minutes at 37°C and then stained with propidium iodide (50 μg/ml). Cell DNA contents (10,000 cells/experimental group) were determined using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) equipped with a ModFit LT program (Verity Software House Inc.), as previously described [28].

8. Nuclear Fractionation

SNU216, SKBr3, and N87 were serum-starved for 24 h and stimulated with EGF (100 ng/ml) for 30 min and then collected for lysis. Nuclear fractions were prepared using NE-PER extraction reagents (Pierce Chemical, Rockford, IL), according to the manufacturer's instructions. Briefly, after removal of the cytoplasmic fraction using cytoplasmic extraction reagents, the insoluble pellet obtained was resuspended in nuclear extraction reagent (100 mmol/L KCl, 10 mmol/L HEPES, pH 7.9, 10% glycerol, 1 mmol/L dithiothreitol, 5 mmol/L MgCl2, 0.1% NP-40, and 10 mmol/L NaF) containing protease inhibitors. After vigorously vortexing every 10 min during incubation on ice for 40 min, the nuclear fraction was isolated by centrifugation. Westernblotting for Lamin B

and α-tubulin was performed to confirm the nuclear fraction and to exclude cytoplasmic contamination, respectively.

9. EGFR and HER2-NLS Mutant Constructs

EGFR-NLS MT (645-47, RRR>AAA) and HER2-NLS MT (667-68, RR>GG) were induced in the cDNA using a QuickChangeTM Site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol, as previously described [29, 30].

10. Luciferase Reporter Assay

After cotransfection with pGL3-TS-Luc and EGFR or HER2 plasmids, the cells were lysed and the TS-luciferase activity was evaluated using a TR717 microplate luminometer (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions. The human TS promoter region was designed as previously described [31]. CMV-β-Gal was also transfected in order to normalize the transfection efficiencies.

11. Chromatin Immunoprecipitation Assay (CHIP)

SNU216 and SKBr3 were serum-starved for 24 h and stimulated with EGF (100 ng/ml) for 30 min. Briefly, cells were cross-linked by addition of 1% formaldehyde for 10 min and glycine was added (125 mmol/L final) for 5 min to stop the cross-linking reaction. Cells were then lysed with a lysis buffer and sonicated. One-tenth of the total chromatin lysate was used for purification of total genomic DNA. The rest of the lysate was used for immunoprecipitation with EGFR or HER2 antibody. After the collection of immunoprecipitates using protein G agarose, protein-DNA complexes were eluted and heated at 65°C to reverse cross-linking. After digesting proteins by proteinase K, DNA fragments were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Either total or immunoprecipitated DNA were analyzed by PCR of 30 or 35 cycles, respectively, at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Specific sequences of the TS promoter in the immunoprecipitates were detected by PCR with primer: forward, 5′- TGGCGCACGCTCTCTAGAGC-3′ and reverse, 5′- GACGGAGGCAGGCCAAGTG-3′. SNU216 and SKBr3 were serum-starved for 24 h and stimulated for 30 min with EGF (100 ng/ml). The primer utilized for the CHIP assay harbors TS essential promoter regions and E2F-1 binding sites [32].

12. Statistics

The statistical significance of the results was calculated by unpaired Student's t test, and P values of <0.05 were considered to be statistically significant.

RESULTS

1. Lapatinib downregulates fluoropyrimidine-target genes including TS

Recently, I reported that lapatinib evidences significant growth inhibitory activity in HER2-amplified gastric cancer (GC) cells and, in combination with 5-FU, results in a synergistic growth-inhibitory effect *in vitro* [33]. In the current study, these *in vitro* findings were confirmed in an *in vivo* context, where it was shown that lapatinib alone or a combination of lapatinib and 5-FU potently inhibited the tumor growth of HER2-amplified N87 GC cell-bearing xenografts (Figure 1.1). These results further support the rationale for a cancer therapy based on a combination of lapatinib and fluoropyrimidine. It has been previously demonstrated that EGFR TKI, such as erlotinib/or gefitinib treatment with fluoropyrimidine, resulted in an synergistic inhibitory effect in non-small-cell lung cancer cells, possibly as the result of TS downregulation via the inhibition of EGFR signaling (10, 11). Accordingly, I have speculated that lapatinib may be superior to EGFR TKI from the standpoint of chemosensitization to fluoropyrimidine. In order to address these issues and investigate the underlying molecular mechanisms, I first conducted a set of

oligonucleotide microarray experiments to compare the effects of gefitinib with lapatinib from the perspective of the chemosensitization to fluoropyrimidine. I utilized three different GC cell lines, SNU216 ($EGFR^{WT}$, $HER2^{Amp}$), which is sensitive to lapatinib (IC_{50} 0.02 μM), or gefitinib (IC_{50} 0.1 μM), SNU484 ($EGFR^{WT}$, $HER2^{WT}$) which is moderately sensitive to lapatinib (IC_{50} m1 μM) and resistant to gefitnib (IC_{50}>10 μM), or SNU668 ($EGFR^{WT}$, $HER2^{WT}$, $K\text{-}ras^{MT}$) which is resistant to both (lapatinib IC_{50}>10 μM, gefitnib IC_{50}>10 μM; Figure 1.2.A, *left*). The expressions of representative nucleotide synthesis-related genes (E2F-1, TS, TK1 (thymidine kinase 1), DHFR(dihydrofolate reductase), RRM2 (ribonucleotide reductase M2 polypeptide), DUT (DUTP pyrophosphatase), NME1 (nuclear diphosphate kinase 1)) were reduced as the result of gefitinib or lapatinib treatment in TKI-sensitive cells. Fold reductions were decreased in proportion with drug sensitivity in these cases. For example, lapatinib downregulated gene expression in lapatinib-sensitive SNU216 and SNU484 cells, but did not affect gene expression in the lapatinib-resistant SNU668. As compared to gefitinib, lapatinib treatment induced higher fold reductions of all of the downregulated genes in SNU216 and SNU 484 cells (Figure 1.2A, *right*). Thus, it is probable that the inhibition of both EGFR and HER2 appears to be more effective from the perspective of

gene regulation. It is also crucial to note that lapatinib modestly downregulates gene expression in HER2 wild-type SNU484 cells, as well as in HER2-amplified cells. The transcription factor E2F-1 downregulates nucleotide synthesis-associated genes, including TS, and the change in E2F-1 as the result of lapatinib treatment was apparent in SNU216 and SNU484 cells, but was not noted in the SNU668 cells. All these data were confirmed via RT-PCR and Western blotting (Figure 1.2B). Lapatinib downregulated TS mRNA and protein in a dose-dependent manner in the SNU216 and SNU484 cells, but not in the SNU668 cells (Figure 1.2B). In HER2-amplified SNU216 cells that were sensitive to both, lapatinib suppressed gene expression more potently than gefitinib. These findings were confirmed in different HER2-amplified N87 and SKBr3 cells following treatment with either gefitinib or lapatinib. As is shown in Figure 1.2C, lapatinib consistently induced a profound reduction of TS, TK1, DHFR, or RRM2 mRNA in a dose-dependent manner as compared to gefitinib. Lapatinib effectively inactivated phosphorylated-EGFR and -HER2 at the same doses as used in Figure 1.2C (Figure 1.2D). Together, these results revealed that lapatinib downregulates a variety of nucleotide synthesis-related genes, including E2F-1 and TS. Moreover, the gene modulation effect of the dual inhibitor is superior to that of

EGFR TKI, which is more apparent in HER2-amplified cells. Considering that these nucleotide synthesis-related genes are major determinants of fluoropyrimidine sensitivity, the dual inhibition of EGFR and HER2 TK by lapatinib appears to represent a promising strategy for the sensitization of cancer cells to fluoropyrimidines in a subset of tumors.

2. Dual inhibition of EGFR and HER2 is required for TS downregulation

In an effort to evaluate the biological and functional relevance of the dual inhibition of EGFR and HER2, I assessed TS protein levels following transfection with small interfering RNA (siRNA) oligonucleotides directed against EGFR, HER2, or both in HER2-amplified SNU216 and SKBr3, wild-type SNU484 cells (Figure 1.3A). The dual inhibition of EGFR and HER2 effectively abolished TS expression in HER2-amplified SNU216, SKBr3, and N87 (data not shown), and similar effects were also observed in the HER2 wild-type cells. Consistent with the results observed with TS downregulation, co-transfection with EGFR and HER2 siRNA induced a profound G1-arrest of cancer cells (Figure 1.3B). These results were confirmed by a pharmacological inhibitor experiment showing that lapatinib significantly

downregulated the TS protein as compared with the effects of gefitinib or trastuzumab in SKBr3 cells (Figure 1.3C). Thus, it can be concluded that the dual inhibition of EGFR and HER2 is required for the more effective downregulation of TS. Even in the HER2 wild-type cells, dual inhibition appears to be superior to single inhibition from the perspective of TS downregulation.

3. Lapatinib inhibits the nuclear translocation of EGFR and HER2

While located in the nucleus, EGFR or HER2 has been shown to function as a transcription factor for DNA repair and synthesis genes (21). In order to determine whether gefitinib or lapatinib influences the nuclear localization of EGFR and HER2, I assessed the cytoplasmic-to-nuclear distribution of EGFR and HER2 following EGF stimulation. In HER2-amplified SNU 216, EGFR was translocated into the nucleus with EGF-stimulation, and HER2 was stably detected both in the cytosol and nucleus. After lapatinib treatment, both nuclear EGFR and HER2 were significantly reduced in these cells, whereas gefitinib treatment induced modest reduction of nuclear EGFR. In contrast, cytosolic EGFR and HER2 were not altered by either gefitinib or lapatinib

(Figure 1.4A). In the case of EGFR, lapatinib appeared to inhibit the ligand-dependent translocation of EGFR into the nucleus. However, as HER2 has already been detected in the absence of ligand in HER2-amplified cells, lapatinib may inhibit preexisting translocated nuclear HER2, independently of the ligand. Although its molecular mechanism will require further investigation, lapatinib appears to reduce levels of nuclear EGFR and HER2, possibly via the inhibition of ligand-dependent or independent translocation of EGFR and HER2.

4. Interaction of nuclear EGFR and HER2 with TS promoter is inhibited by lapatinib

I subsequently assessed the effects of nuclear EGFR and HER2 on TS gene transcription. First, I compared TS promoter activity according to the transfection of EGFR and HER2 nuclear localization signal (NLS) WT or MT in both EGFR and HER2-negative CHO cells. Following transient transfection with EGFR-NLS WT, HER2-NLS WT or both, I noted that TS promoter activity was increased to a greater degree by forced HER2 expression than by forced EGFR expression, and was activated more profoundly by dual-expression

than by single-expression. By way of contrast, after transfection with EGFR-NLS MT, HER2-NLS MT, or both, TS promoter activation was lower than the effects of WT (Figure 1.4B). Consistently, the knockdown of EGFR and/or HER2 and lapatinib treatment significantly reduced the ability of the TS promoter in response to EGF in SKBr3 cells, as compared to what was observed with gefitinib or trastuzumab alone (Figure 1.4C). CHIP demonstrated that EGF-induced nuclear EGFR and HER2 bind to the TS promoter in SNU216 and SKBr3 cells, and that their association was abolished by lapatinib (Figure 1.4D). Collectively, these results reveal that nuclear EGFR and HER2 activate TS gene transcription via binding to the TS promoter, and that the TS promoter-bound EGFR and HER2 are inhibited by lapatinib, ultimately resulting in the downregulation of TS.

Figure 1.1. Combination of lapatinib and 5-FU potently inhibited tumor growth of N87-bearing xenografts.

N87 cells (5×106) were injected s.c. into nude mice with randomization (n = 6). Treatment with lapatinib (100 mg/kg, p.o., daily for 3 weeks) and 5-FU (50 mg/kg, i.p., once weekly for 3 weeks) was initiated once the tumors had achieved a volume of 50–100 mm3. Bars, SEM and repeated measures of ANOVA showed statistically significant effects (P<0.005) in the lapatinib and combination groups.

Figure 1.1.

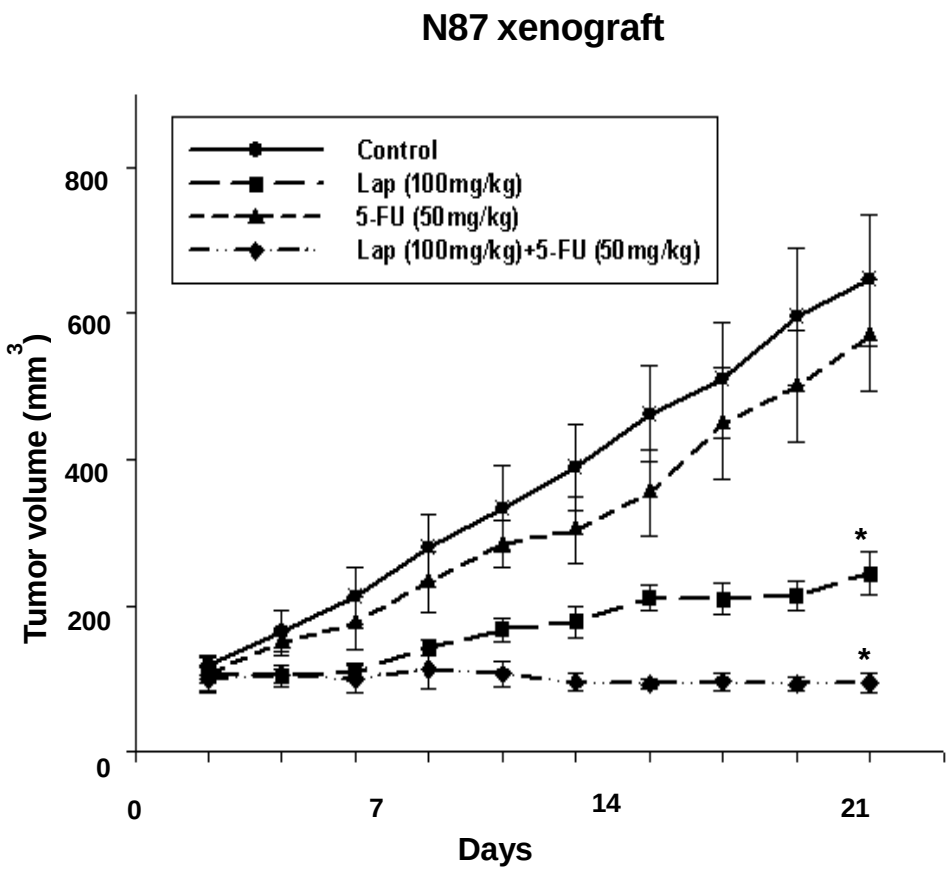

N87 xenograft
Control
Lap (100mg/kg)
5-FU (50mg/kg)
Lap (100mg/kg)+5-FU (50mg/kg)
Tumor volume (mm3)
800
600
400
200
0
0
7
14
21
Days
*
*

Figure 1.2. Lapatinib downregulates fluoropyrimidine-target genes.

A, Downregulated fluoropyrimidine-sensitivity genes by oligonucleotide microarray based on analysis comparing DMSO/lapatinib-treatment versus DMSO/gefitinib-treatment samples at 24 hours of each treatment in SNU216, SNU484, or SNU668 cells. B, The indicated cells were treated with lapatinib (0.1, 1, 1 μmol/L) for 24 hours. mRNA levels of E2F1 and TS were assessed via RT-PCR (upper). β-Actin was utilized as a loading control. Western blots are provided for E2F1 and TS (lower). α-tubulin was employed as a loading control. C and D, N87, and SKBr3 cells were grown for 24 hours in the presence of gefitinib (0.01, 0.1, 1 μmol/L) or lapatinib (0.01, 0.1, 1 μmol/L). Western blots are shown for phosphorylated and total EGFR, and HER2. mRNA levels of TS, TK1, DHFR, and RRM2 were determined via quantitative real-time RT-PCR. Columns, means; bars, ±SD. *, $P<0.05$; **, $P<0.05$;***, $P<0.05$, gefitinib versus lapatinib at 0.01, 0.1, and 1 μmol/L doses. The expressed data are representative of three independent experiments.

Figure 1.2A.

	IC_{50} (μM)	
	Gefitinib	Lapatinib
SNU 216	0.1±0.004	0.02±0.003
SNU 484	>10	1±0.014
SNU 668	>10	>10

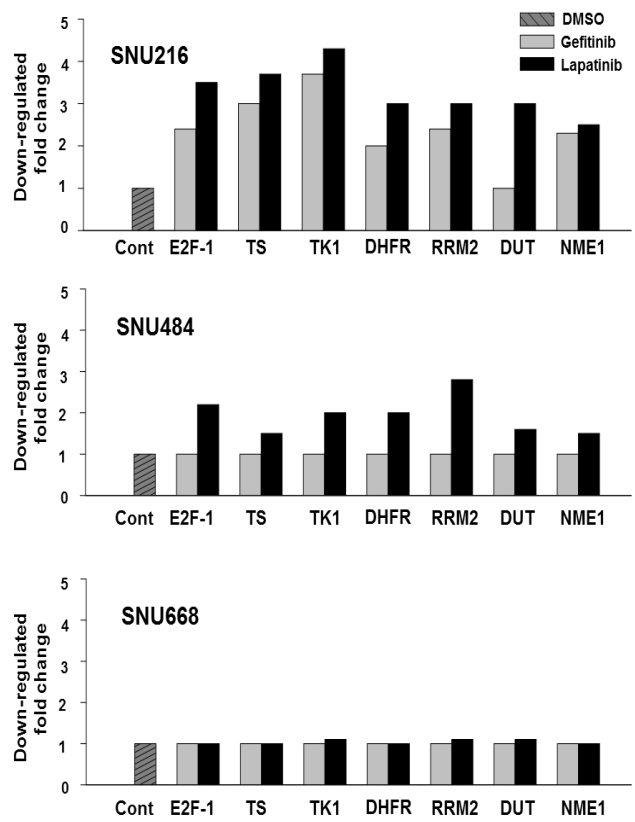


Figure 1.2B.

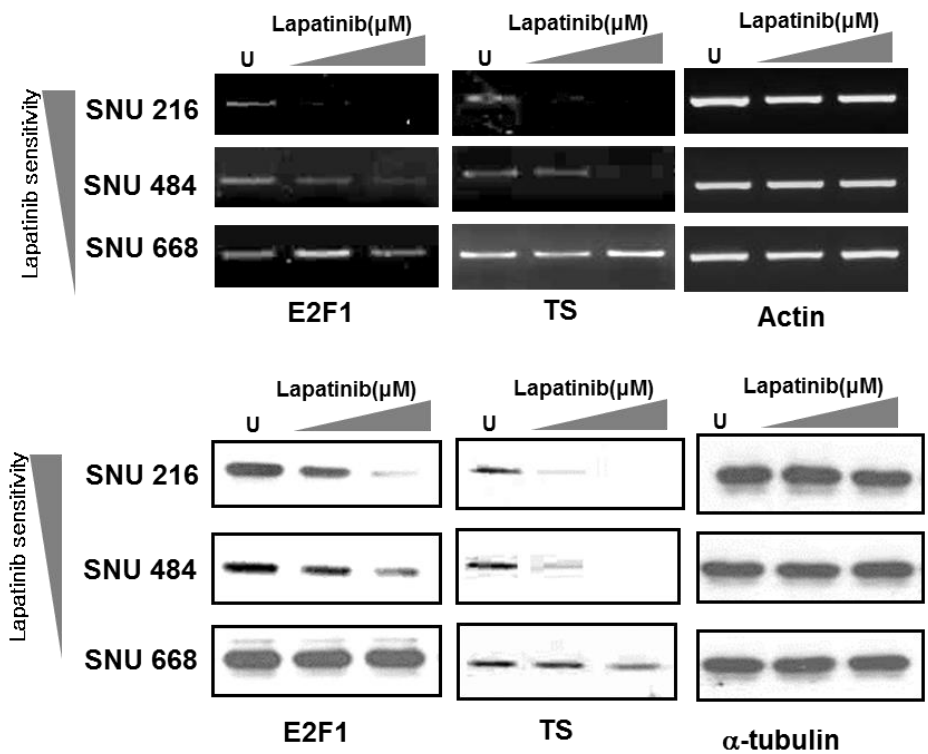

Lapatinib(μM)
U
Lapatinib sensitivity
SNU 216
SNU 484
SNU 668
E2F1
TS
Actin
α-tubulin

Figure 1.2C.

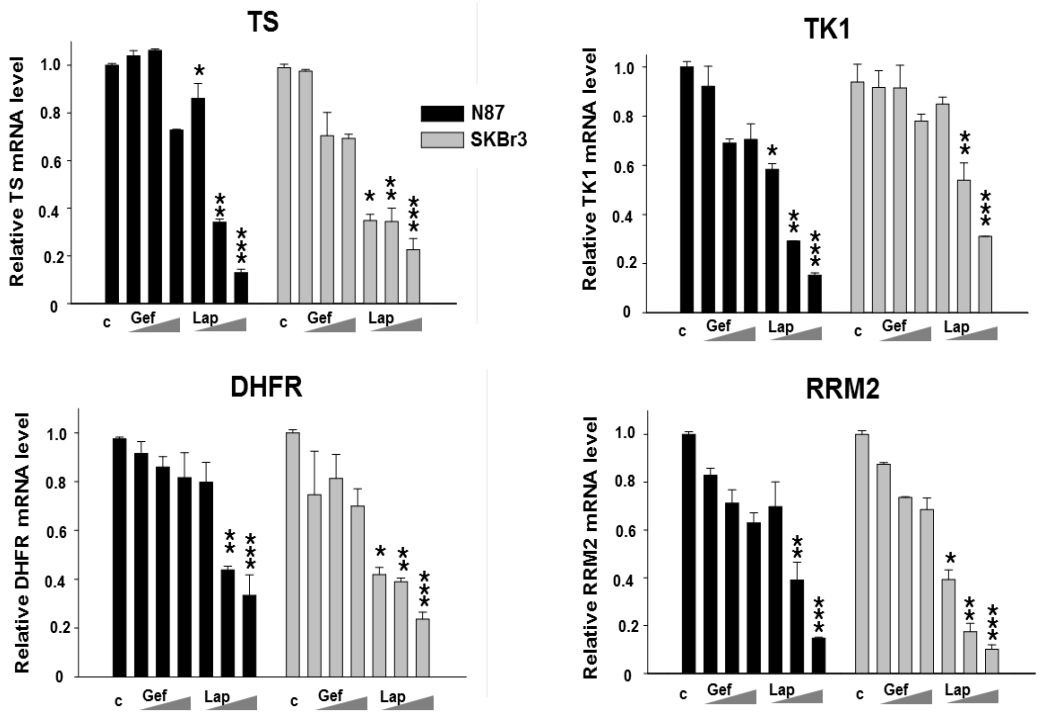

TS
Relative TS mRNA level
N87
SKBr3
TK1
Relative TK1 mRNA level
DHFR
Relative DHFR mRNA level
RRM2
Relative RRM2 mRNA level
c
Gef
Lap

Figure 1.2D.

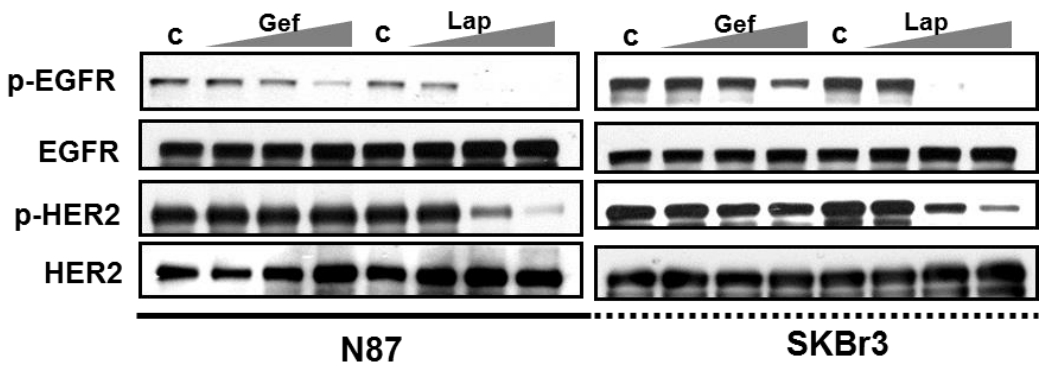


Figure 1.3. Dual inhibition of EGFR and HER2 kinase activity is required for TS downregulation.

A, SiRNAs targeting endogenous EGFR, HER2, and both missense transcripts were transfected with SNU216, SKBr3, and SNU484 prior to 48 hours of lysate treatment. Whole cell extracts were Western-blotted with EGFR, HER2, and TS. α-tubulin was utilized as a loading control. B, The cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to flow cytometric analysis. Proportions of cells in the G1, S, and G2-M phase were quantified using the ModFit LT program (Verity Software House Inc.); total percentages of G1, S, and G2-M phases are 100% in our data. C, SKBr3 cells were exposed for 24 h to gefitinib (1 μmol/L), trastuzumab (100 ng/ml), or lapatinib (1 μmol/L). Western blots are provided for TS. α-tubulin was used as a loading control. The expressed data are representative of three independent experiments.

Figure 1.3A.

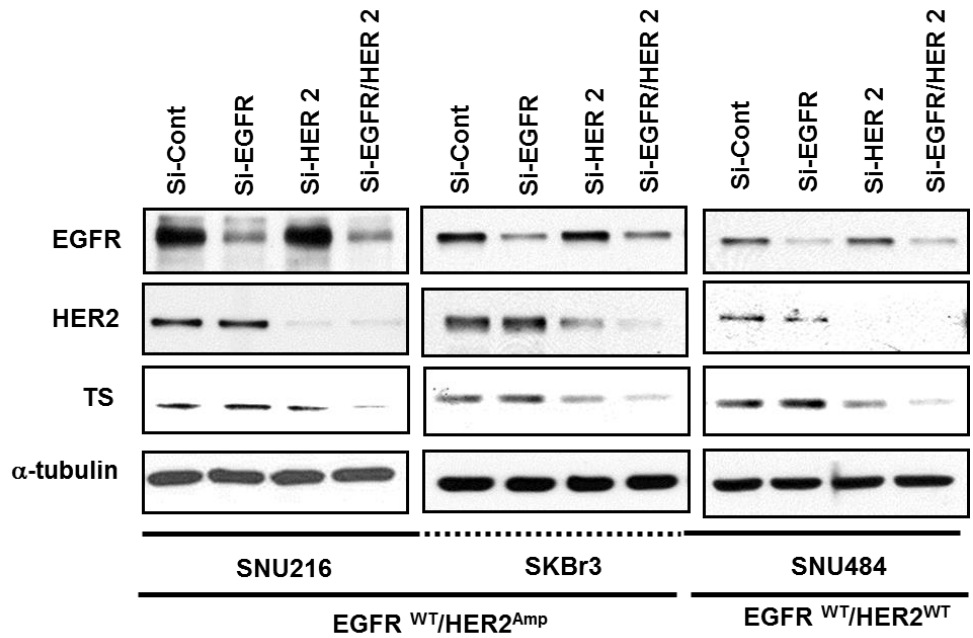

Si-Cont
Si-EGFR
Si-HER 2
Si-EGFR/HER 2
Si-Cont
Si-EGFR
Si-HER 2
Si-EGFR/HER 2
Si-Cont
Si-EGFR
Si-HER 2
Si-EGFR/HER 2
EGFR
HER2
TS
α-tubulin
SNU216
SKBr3
SNU484
EGFR WT/HER2Amp
EGFR WT/HER2WT

Figure 1.3B

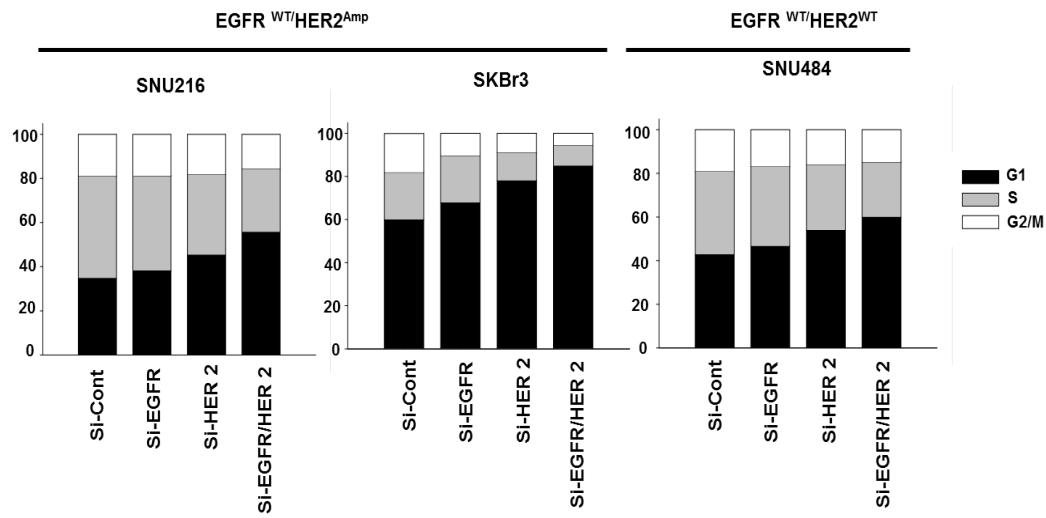

EGFR WT/HER2Amp
EGFR WT/HER2WT
SNU216
SKBr3
SNU484
100
80
60
40
20
0
Si-Cont
Si-EGFR
Si-HER 2
Si-EGFR/HER 2
G1
S
G2/M

Figure 1.3C.

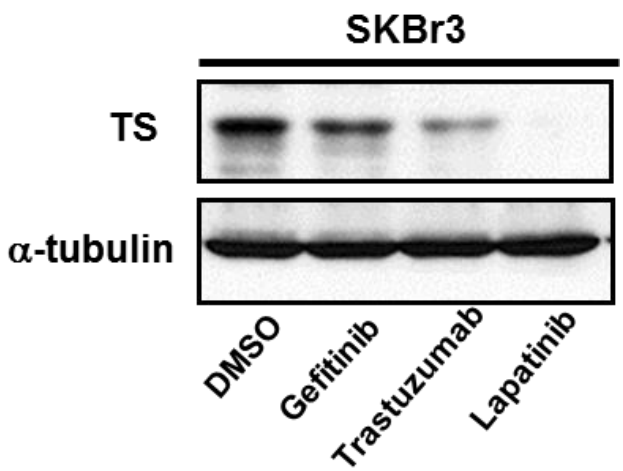


Figure 1.4. TS gene transcription by nuclear EGFR and HER2 is inhibited by lapatinib.

A, Prior to harvesting, SNU216 cells were serum-starved for 24 hours, then grown for 3 h in the presence of gefitinib and lapatinib (0.1, 1 μmol/L), followed by 30 minutes of EGF (100 ng/mL) stimulation, then subjected to biochemical fractionation to separate the nucleus from the cytosolic material. Western blots are shown for EGFR and HER2. The loading controls were Laminin B (nucleus marker) and α-tubulin (cytosol marker). B, EGFR- and HER2-negative CHO cells were co-transfected with TS-promoter luciferase construct and plasmids encoding for EGFR-NLS WT/MT, HER2-NLS WT/MT, or both, in addition to mock plasmid. Following 24 hours of serum-starvation, the cells were stimulated for 6 hours with EGF and subjected to luciferase assays. C, TS-promoter luciferase construct and Si-RNAs targeting endogenous EGFR, HER2, and both missense transcripts were transfected with SKBr3, prior to 48 hours of lysate treatment (left). The cells transfected with TS-promoter luciferase construct were pretreated with gefitinib (1 μmol/L), trastuzumab (100 ng/ml), or lapatinib (1 μmol/L) for 24 hours (right). Luciferase activity was then determined. Columns, means; bars, ±SD. D, SNU216 and SKBr3 cells were serum-starved for 24 hours, then grown in the

presence of lapatinib (1 μmol/L) for 3 hours, followed by 30 minutes of EGF (100 ng/mL) stimulation, and then subjected to CHIP analysis. Immunoprecipitation was then conducted with anti-EGFR, anti-HER2, or normal rabbit IgG. The TS promoter region which contained essential promoter regions and E2F-1 binding sites was PCR-amplified. Input nuclear DNA were utilized as a PCR control. The expressed data are representative of three independent experiments.

Figure 1.4A.

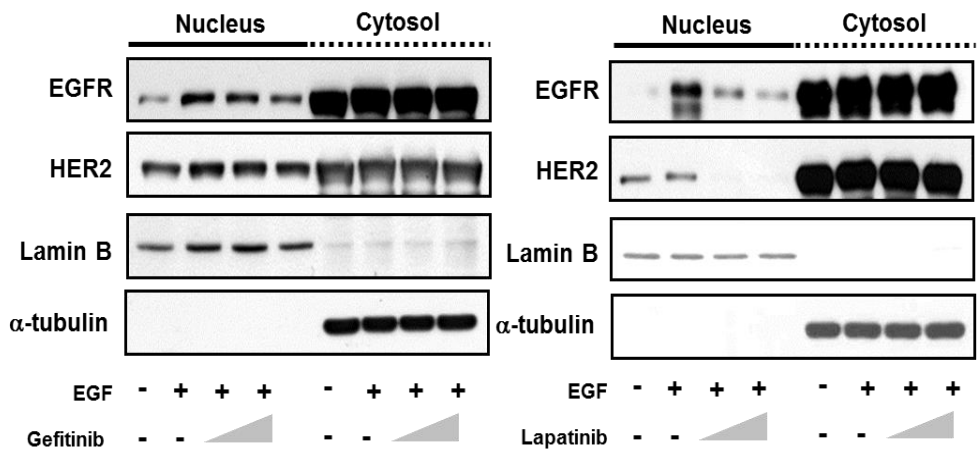

Nucleus
Cytosol
EGFR
HER2
Lamin B
α-tubulin
EGF - + + + - + + +
Gefitinib - - - -
Nucleus
Cytosol
EGFR
HER2
Lamin B
α-tubulin
EGF - + + + - + + +
Lapatinib - - - -

Figure 1.4B.

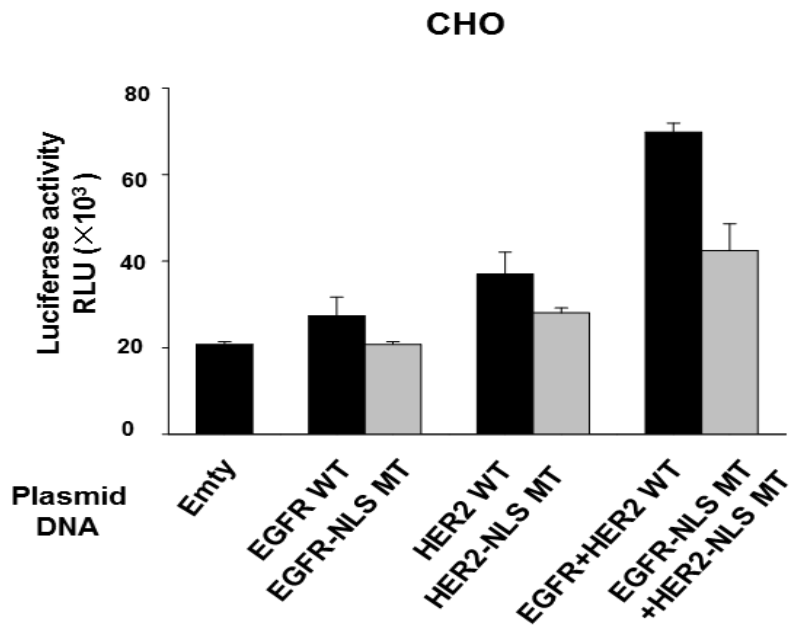

CHO
Luciferase activity
RLU (×10³)
80
60
40
20
0
Plasmid
DNA
Emty
EGFR WT
EGFR-NLS MT
HER2 WT
HER2-NLS MT
EGFR+HER2 WT
EGFR-NLS MT
+HER2-NLS MT

Figure 1.4C.

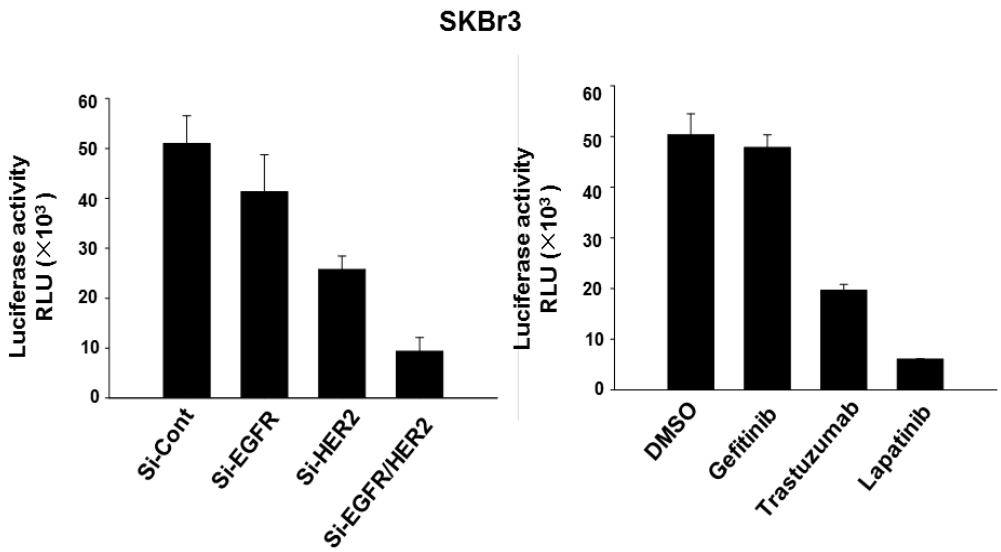
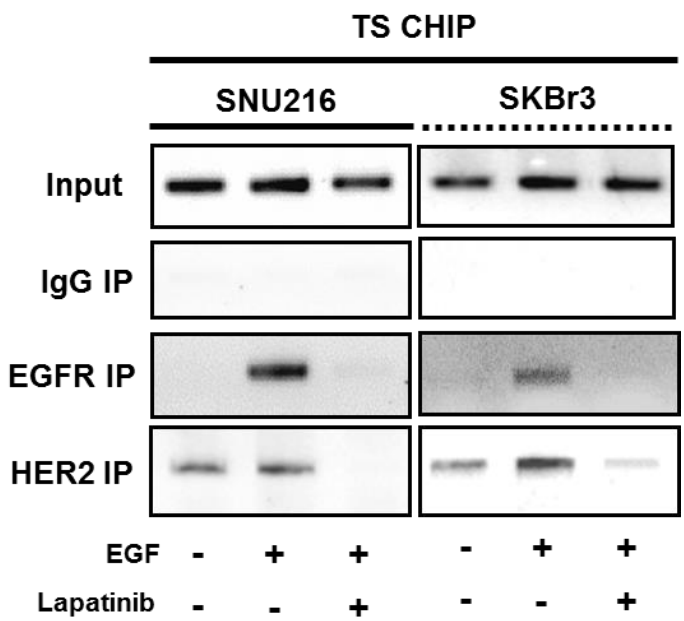

SKBr3
Luciferase activity
RLU (×10³)
60
50
40
30
20
10
0
Si-Cont
Si-EGFR
Si-HER2
Si-EGFR/HER2
Luciferase activity
RLU (×10³)
60
50
40
30
20
10
0
DMSO
Gefitinib
Trastuzumab
Lapatinib

Figure 1.4D.


TS CHIP
SNU216
SKBr3
Input
IgG IP
EGFR IP
HER2 IP
EGF - + + - + +
Lapatinib - - + - - +

DISCUSSION

In this study, I have demonstrated that lapatinib, a dual inhibitor of EGFR and HER2 TK, effectively downregulates a variety of nucleotide synthesis-related genes, including TS, and exhibits activity superior to that of gefitinib not only in HER2-amplified cells, but also in wild-type cells. As a mechanism, I have determined, for the first time, that nuclear EGFR and HER2 activate TS gene transcription, and that EGFR and HER2-bound TS promoter activities are inhibited by lapatinib treatment.

Traditionally, EGFR and HER2, once activated, form a homo- or heterodimer and transduce the mitogenic signal into the nucleus . However, it has also been demonstrated that ligand-activated EGFR or overexpressed HER2 can form a homo- or heterodimer, and that these are also translocated into the nucleus. Therefore, nuclear EGFR and/or HER2 can operate as transcription factors, activating a variety of genes, including iNOS, B-MYB, and COX-2. Considering that nuclear EGFR and HER2 can function as transcription factors, it can be plausibly surmised that other genes involved in cell proliferation might also be regulated by nuclear EGFR and HER2. In the

current study, I demonstrated that TS is activated by nuclear EGFR and HER2. In the context of TS activation, it appears that EGFR and HER2 may perform a pivotal role, as the dual inhibitor, lapatinib, or the double knockdown of EGFR and HER2, evidenced the maximal TS inhibitory effects. It is also worth noting that lapatinib is superior to gefitinib with regard to gene regulation, which also emphasizing the significance of the dual inhibition of EGFR and HER2.

In SNU216 cells evidencing HER2 overexpression, I determined that nuclear EGFR and HER2 are induced in a ligand-dependent and -independent fasion, respectively. The nuclear translocation of EGFR upon ligand stimulation is noticeable in these cells and is inhibited by lapatinib, which implies that the inhibition of EGFR is also required in HER2-amplified cells. It is also conceivable that nuclear EGFR and HER2 may form a homo- and heterodimer, which would bind to the promoters of target genes such as TS. I have previously determined that lapatinib inhibits the heterodimer of EGFR and HER2 in cancer cells. Because lapatinib reduced the levels of nuclear EGFR and HER2, I speculated that lapatinib might also destabilize the levels of hetero- or homodimers of EGFR and HER2 in the nucleus. In HER2 wild-type cells, lapatinib exerted modest gene downregulation effects,

as is shown in Figures. 1.2A and 2B. Therefore, it appears that lapatinib can be tested for the purpose of gene modulation, regardless of the HER2 status.

Collectively, the data provided in this study demonstrate the molecular mechanisms underlying the synergy between lapatinib and fluoropyrimidine. In the future, lapatinib should be investigated as a chemosensitizing agent that enhances cytotoxicity or circumvents resistance against anticancer drugs, not only in HER2-amplified, but also in HER2 wild-type tumors**.**

Ⅲ. Combined lapatinib and cetuximab enhance cytotoxicity against gefitinib-resistant lung cancer cells

ABSTRACT

Although non-small cell lung cancer (NSCLC) cells with somatic mutations in their epidermal growth factor receptors (EGFR) initially show a dramatic response to tyrosine kinase inhibitor (TKI), these cells eventually develop resistance to TKI. This resistance may be caused by a secondary T790M mutation in the EGFR tyrosine kinase, which leads to the substitution of methionine for threonine in 790. In this study, I show that a combination of lapatinib and cetuximab overcomes gefitinib resistance in NSCLC with the T790M mutation. I observed that T790M lung cancer cells were resistant to gefitinib, and Stat3 was persistently activated in the resistant cells. A reversible EGFR and HER2 TKI, lapatinib, decreased Stat3 activation by blocking heterodimerization of EGFR and HER2, which led to a modest increase in the inhibitory effect on gefitinib-resistant T790M cells. In addition to lapatinib, the anti-EGFR antibody, cetuximab, induced down-regulation of EGFR and apoptotic cell death in T790M cells. Finally, combined lapatinib and cetuximab treatment resulted in significantly enhanced cytotoxicity against gefitinib-resistant T790M cells in vitro and in vivo. Taken together, these data suggest that treatment with a combination of lapatinib and cetuximab, which

induces dimeric dissociation and EGFR down-regulation, appears to be an effective strategy for treatment of patients with EGFR TKI-resistant NSCLC.

INTRODUCTION

Epidermal growth factor (EGF) receptor (EGFR) is a major target of molecular anticancer therapy. Two treatment approaches involving the use of monoclonal antibodies and receptor tyrosine kinase inhibitors (TKI) have been developed, and both of these approaches have shown benefits in clinical trials [34]. Although anti-EGFR therapies are active in some patients, in nearly all patients, the disease eventually becomes resistant to therapy. Therefore, a better understanding of the mechanisms underlying the resistance to anti-EGFR therapies is critical to improve the efficacy of these treatments. The mechanisms that mediate resistance to anti-EGFR therapies include autocrine and paracrine production of ligands, secondary mutations, the constitutive activation of downstream pathways, and the activation of alternative pathways such as angiogenesis [35-37]. Recent studies have shown that a single secondary mutation that results in the substitution of methionine for threonine at position 790 in exon 20 (T790M) confers resistance to gefitinib in lung cancer cells [37]. The T790M mutation is observed in ~50% of patients whose disease progresses after an initial response to gefitinib or erlotinib. It is suggested that the T790M mutation

confers resistance by inducing steric hindrance of erlotinib binding as a result of the presence of a bulkier methionine side chain in the ATP kinase binding pocket [37]. This T790M mutation is structurally analogous to other TKI-resistant mutations, such as T351I in Bcr-Abl and T670I in c-Kit, which also cause steric hindrance resulting in the inhibition of TKI binding [38-40]. Besides T790M, recent studies have shown that amplification of c-MET also confers the resistance to EGFR TKI [41].

Elucidation of the molecular mechanisms leading gefitinib or erlotinib resistance has led to the development of alternative strategies that can circumvent resistance to EGFR TKIs. These strategies have included development of a second generation of EGFR TKIs that include EKB-569 and CL-387,785 (which inhibit EGFR), HKI-272, and BIBW-2992 (which inhibits HER2 as well as EGFR) [42-44]. Unlike first-generation EGFR TKIs, most second-generation TKIs are irreversible inhibitors that form a stable covalent bond with cysteine residues present in EGFR or HER2. Based on their irreversible and specific binding activities, previous studies have suggested that irreversible TKIs may be able to overcome the gefitinib or erlotinib resistance caused by T790M [45]. Although it is unclear if the inhibition of HER2 in addition to that of EGFR plays a role in overcoming the resistance,

inhibitors with dual activity may have therapeutic advantages over compounds that only inhibit EGFR because heterodimerization of EGFR and HER2 has a greater effect on EGF signal activation than EGFR homodimerization.

Lapatinib (GW572016, Tykerb) is a small molecule that is administered orally and functions as a dual reversible inhibitor of EGFR and HER2 tyrosine kinases [46]. Lapatinib has been shown to significantly inhibit the proliferation of cancer cells showing EGFR and/or HER2 overexpression both in vitro and in vivo [5, 6, 47]. However, the effects of lapatinib on lung cancer are largely unknown. Cetuximab (Erbitux) is a chimeric monoclonal antibody that competes with receptor ligands for binding to EGFR [47, 48]. Cetuximab undergoes internalization and, in some cells, induced receptor degradation and down-regulation [49-51]. Recent studies have shown that, when combined with gefitinib or erlotinib treatment, cetuximab is also effective in cancer cells expressing mutant EGFR. Moreover, treatment with a combination of gefitinib and cetuximab has shown a synergistic effect against EGFR-dependent tumor cells both in vitro and in vivo, suggesting that combined treatment with these drugs causes a complete blockade of EGF signaling [52, 53].

In this study, I show that active heterodimerization of EGFR and HER2 is

blocked by lapatinib and that EGFR is down-regulated by cetuximab in gefitinib-resistant T790M lung cancer cells. These findings indicate that combined treatment with lapatinib and cetuximab results in enhanced cytotoxicity against T790M cells in vitro and in vivo. Given that receptor dimerization and EGFR expression are the main signals on EGF activation, treatment with a combination of lapatinib and cetuximab may be an effective strategy for EGFR targeted therapy**.**

MATERIAL AND METHODS

1. Tissue Procurement and Mutational Analyses of the EGFR Gene

Tumor specimens obtained before gefitinib treatment and at the time that progression of the disease occurred despite gefitinib treatment were obtained by fine-needle biopsies. The protocols used in this study were approved by the Institutional Review Board of Seoul National University Hospital and informed consent was received from all patients included in this study. Genomic DNA was extracted from the tumor specimens, and EGFR (exons 18-24) was then sequenced using primers and methods as described previously [54]. All sequencing reactions were done in both forward and reverse directions, and all mutations were confirmed at least twice from independent PCR isolates as described previously [54].

2. Cell Culture and Reagents

H1975 human cancer cells and COS7 monkey kidney cells were grown at 37°C under 5% CO2 in either RPMI 1640 or DMEM containing 10% fetal

bovine serum (WELGENE, Inc.). H1975 and COS7 cells were purchased from American Type Culture Collection. Gefitinib was a gift from AstraZeneca, and lapatinib was a gift from GlaxoSmithKline. Cetuximab and trastuzumab were kindly provided by Merck and Roche, respectively. Stock solutions were prepared in DMSO, and then stored at -20°C. Gefitinib and lapatinib were diluted in fresh medium before each experiment, and the final concentration of DMSO was <0.1%. EGF was purchased from Sigma-Aldrich.

3. EGFR Mutant Constructs and Transfection

The cDNA for human EGFR was kindly provided by Dr. Y. Yarden (Weizmann, Institute of Science). Point mutations of E709K, G719A, T790M, L858R, A859T, or T790M/L858R were induced in the cDNA using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Each mutant construct was confirmed by sequencing and then transfected into the COS7 cell line using LipofectAMINE 2000 to generate stable cell lines after selection with G418 following the method described by the manufacturer (Invitrogen).

4. Western Blotting and Immunoprecipitation

Antibodies against phosphorylated EGFR (pY858 and pY1068), phosphorylated signal transduction and activation of transcription 3 (Stat3; pY705), phosphorylated Akt (pS473), phosphorylated mitogen-activated protein kinase (MAPK; Thr202/Tyr204), HER2, Stat3, Akt, and MAPK were purchased from Cell Signaling Technology. Anti-EGFR, poly(ADP-ribose) polymerase, caspase-3, cyclin D, cyclin E, cyclin A, cyclin B, p27, and p21 antibody were obtained from Santa Cruz Biotechnology. Cultured cells that had reached ～70% to 80% confluence were used for protein analyses. To analyze the EGFR signaling in various mutants, cells were starved for 24 h, and then treatment drugs were added for 3 h followed by stimulation with EGF (100 ng/mL) for 30 min before cell harvest. For the analysis of apoptosis signaling as a result of drug treatments, cells were treated with different concentrations of lapatinib or cetuximab for 24, 48, or 72 h. Cells were then lysed in RIPA or NP40 to induce immunoprecipitation following a previously described method [55] and then analyzed by Western blotting using an enhanced chemiluminescence system.

5. Growth Inhibition Assay

The viability of cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) assays. Briefly, cells were seeded in complete growth medium in 96-well plates at a density of 3,000 per well (COS7-L858R/T790M and H1975). Following overnight incubation, cells were grown for 24 h in medium supplemented with or without 0.1%, 1%, and 10% serum and/or EGF and then incubated for an additional 72 h in the presence of the treatment drugs.

6. Annexin V Staining

H1975 cells were treated with 0.5 and 5 μmol/L lapatinib and 1 and 10 μg/mL cetuximab for 72 h, at which point cells were collected and stained with Annexin V-phycoerythrin and 7-aminoactinomycin D (Becton Dickinson). Apoptotic cell death was measured by counting the number of cells that stained positive for Annexin V-phycoerythrin as assessed by fluorescence-activated cell sorting analysis.

7. H1975 Xenografts

Animal experiments were carried out in the animal facility of the Seoul National University in accordance with institutional guidelines. Six- to 8-week-old female BALB/c athymic (nu+/nu+) mice were purchased from Central Lab Animal, Inc. (Seoul, Korea). The initial body weight of the animals at the time of arrival was between 18 and 20 g. Mice were allowed to acclimatize to local conditions for 1 week before being injected with cancer cells. Tumors were induced by injecting H1975 cells (5 × 106) subcutaneously into the right flank of mice. The tumors were then measured twice a week using calipers, and the tumor volume (mm3) was calculated according to following formula: ((width2 × height) / 2). When tumors had reached a volume of 50 to 100 mm3, treatment with either gefitinib, lapatinib, cetuximab, a combination of lapatinib and cetuximab, or a vehicle control was initiated. Gefitinib and lapatinib were administered via oral gavage at a concentration of 50 to 150 mg/kg in 0.5% Tween80 (Sigma) in sterile milli-Q water Monday to Friday for 3 weeks. A dose of 10 mg/kg of cetuximab was given i.p. twice a week. Xenograft tumors were excised from euthanized mice and snap frozen in liquid nitrogen, and the lysates were then prepared in RIPA using a Dounce homogenizer. Total protein levels were determined, and detection of the EGFR levels was

assessed by Western blot as described previously [56].

8. Statistical Analysis

An unpaired two-tailed t test was used to determine the significance of change in levels of cell viability and apoptosis between different treatment groups. Statistical analysis to compare tumor sizes in xenograft-bearing mice was done with ANOVA. Differences between groups were considered statistically significant if $P < 0.05$.

RESULTS

1. Secondary T790M mutation confers resistance to gefitinib-sensitive L858R mutant cells

I identified a patient with a T790M secondary mutation in the EGFR TK domain. This patient with a L858R mutation initially responded to gefitinib, however, after 8 months of treatment her symptoms worsened and a CT scan showed that the disease had progressed. Tumor tissues obtained at the time of progression confirmed the presence of adenocarcinoma, and EGFR sequencing revealed an additional C-to-T mutation at nucleotide 2369 of exon 20, indicating a T790M mutation. In order to test and compare the sensitivity of T790M cells to gefitinib, various EGFR mutants, including T790M mutants, were derived from NSCLC patients. L858R mutants were included as a gefitinib-sensitive mutation to provide a reference, whereas E709K, G719A, and A859T mutants were derived from patients that did non respond to gefitinib [54]. Next, each of these mutants was transfected into COS7 cells, which have low levels of endogenous EGFR (Figure 2.1A). Because these EGFR TK mutations reside near the ATP cleft, where gefitinib binds, it was

expected that these mutant cells would show altered sensitivity to EGFR inhibitors. EGF-induced autophosphorylations of EGFR were measured in cells pretreated with various concentrations of gefitinib. When the sensitive L858R cells were compared to cells containing the resistant E709K, G719A, and A859T mutations, a descending level of sensitivity to gefitinib was observed, with the least amount of sensitivity being observed in cells containing the T790M mutation. All mutants, with the exception of the T790M mutant, were found to be completely inhibited by gefitinib at a concentration of 100 nmol/L.

To confirm the functional effect of the EGFR T790M mutation, COS7 cells were stably transfected with wild type, L858R, T790M or a combination of L858R and T790M DNA. In the absence of gefitinib, cells with either mutant expressed similar levels of total EGFR and phosphorylated EGFR, which suggests that the presence of the T790M mutation does not substantially alter the production, degradation, or activation of the scaffold EGFR molecule. Moreover, following treatment with gefitinib, cells with either the T790M mutation alone or both the L858R and T790M mutations showed persistent EGFR activation (Figure 2.1B). Similarly, T790M-transfected PC9 cells with an in-frame deletion of exon 19 (delE746-A750) showed persistent EGFR

activation after gefitinib treatment (data not shown). Consistent with our observations, the T790M mutation has been reported in patients resistant to gefitinib and erlotinib therapy. To better understand how T790M induces gefitinib resistance at a molecular level, I examined the effects of gefitinib on Stat3, Akt, and MAPK, the main downstream signaling effectors of EGFR. Following gefitinib treatment, phosphorylated Akt and MAPK levels were not significantly different between gefitinib-sensitive cells containing the L858R mutation and resistant cells containing the T790M mutation or both the L858R and T790M mutations. Conversely, phosphorylation of Stat3 was not inhibited by gefitinib in cells containing the T790M mutation or cells containing both the L858R and T790M mutations, whereas it was blocked in cells transfected with only the gefitinib-sensitive L858R mutation (Figure 2.1C). Additionally, transfected PC9 cells containing the delE746-A750 mutation showed persistent Stat3 phosphorylation after gefitinib treatment, similar to what was observed in the T790M transfected cells (data not shown). Together, these data suggest that the secondary T790M mutation confers resistance to gefitinib-sensitive L858R mutant lung cancer cells via persistent Stat3 activation.

2. Lapatinib inhibits the heterodimerization of EGFR and HER-2, which

leads to a growth inhibitory effect on gefitinib-resistant H1975 cells

Next, because Stat3 is a major downstream signal of EGFR activation, I speculated that the constitutively activated ligand-receptor interaction might contribute to gefitinib resistance. Based on this speculation, I screened several EGFR targeting agents that can affect ligand-receptor interactions. I found that, compared to gefitinib or trastuzumab, lapatinib significantly inhibited the phosphorylation of Stat3 in H1975cells that harbored both the L858R and T790M mutations of the EGFR (Figure 2.2A). I also noticed that cetuximab modestly decreased Stat3 phosphorylation. It was previously reported that Stat3 is a critical mediator of the oncogenic effects of EGFR mutation in NSCLC among the various downstream signals following EGFR activation [57]. Our data also support that Stat3 is one of key signals in gefitinib-resistant T790M cells.

Next, I used an MTT assay to determine if lapatinib was able to inhibit the growth of stably transfected COS7-L858R/T790M cells and H1975 cells (Figure 2.2B). The IC50 of lapatinib was between 1-2 μmol/L , and cells treated with lapatinib showed enhanced cytotoxicity compared to those treated with gefitinib. Consistent with the results of the in vitro experiment, lapatinib treatment delayed tumor growth in H1975 tumor-bearing mice

(Figure 2.2C).

I then attempted to determine the mechanism by which lapatinib inhibits the phosphorylation of Stat3 in gefitinib-resistant cells. Gefitinib is known to induce the dimeric dissociation or inactive dimerization of receptors by inhibiting EGFR TK activity [58]. In gefitinib-resistant T790M cells, I found that the heterodimerization of EGFR and HER2 was maintained in the presence of gefitinib, which resulted in the persistent activation of Stat3 (Figure 2.2D). Conversely, lapatinib treatment significantly reduced the heterodimerization of EGFR and HER2, which subsequently diminished the phosphorylation of tyrosine residues. Therefore, it is likely that the activation of Stat3 in gefitinib-resistant T790M cells is mediated by active heterodimerization of EGFR and HER2, which is effectively blocked by lapatinib. Phosphorylation of Akt and MAPK were also significantly inhibited by treatment with 1 μmol/L of lapatinib.

3. Cetuximab in combination with lapatinib enhances cytotoxicity against T790M NSCLC

I next performed cell growth inhibition with gefitinib or lapatinib by

culturing cells with or without serum and/or EGF to examine whether inhibition of ligand binding affects cell growth in H1975 cells. The maximum growth inhibition was observed when cells were grown with lapatinib in the absence of serum and EGF, which indicates that T790M H1975 cells may partially retain ligand-dependent growth (Figure 2.3A). These data prompted us to test the combination effect of treatment with anti-EGFR antibody to cetuximab since cetuximab competes for binding with the ligand to EGFR. In experiments comparing the cell growth inhibition of lapatinib treated cells and cells treated with a combination of lapatinib and cetuximab, I observed that the growth inhibition effects on H1975 cells was further inhibited in a dose-dependent manner (Figure 2.3B, left). However, incubation with gefitinib and cetuximab left H1975 cells unaffected by signle agent as well as combination treatment. Annexin V staining showed that lapatinib alone induced apoptotic cell death in a dose-dependent manner, but cetuximab did not. However, treatment with a combination of lapatinib and cetuximab resulted in a greater induction of apoptosis. For example, treatment with 0.5 μmol/L of lapatinib and 5 μg/mL of cetuximab induced apoptosis in 18% of cells, whereas treatment with lapatinib or cetuximab alone resulted in apoptosis in 6.5 and 4% of cells, respectively (Figure 2.3B, right).

To further confirm the previous data, I performed western blotting to determine the level of phosphorylation and total EGFR, Stat3, Akt, and MAPK (Figure 2.3C). In terms of EGFR inhibition, lapatinib tended to inhibit phosphorylated EGFR, whereas cetuximab significantly decreased the level of EGFR. Treatment with a combination with lapatinib and cetuximab significantly decreased both the phosphorylated EGFR and total EGFR. Activated downstream signals, including Stat3, were also significantly inhibited by treatment with a combination of lapatinib and cetuximab. In addition, PARP cleavage and activated caspase 3 were increased as a result of treatment with a combination of lapatinib and cetuximab, which is consistent with the results of the apoptotic assay. The cell proliferation-related cyclins D, E, A and B were all decreased after treatment with the combination therapy, whereas cell cycle inhibitory p21 and p27 were increased after treatment with a combination of lapatinib and cetuximab. Finally, I tested the effects of treatment with a combination of lapatinib and cetuximab on gefitinib-resistant H1975 xenografts that expressed the L858R/T790M mutant EGFR (Figure 2.3D, left), and found that treatment with a combination of lapatinib and cetuximab completely inhibited tumor growth in the H1975 bearing xenograft. The growth inhibition effect of cetuximab alone was also notable

and appeared to be greater than that of lapatinib alone. Since the cetuximab-induced anti-tumor effect may involve an immune mechanism unrelated to the inhibition on EGFR, I tested the effect of drug treatment on EGFR and downstream molecules. Treatment with cetuximab alone reduced phosphorylated and total EGFR; however phosphorylated and total EGFR were more reduced as a result of combination treatment in H1975 bearing xenografts (Figure 2.3D, right), suggesting that the antitumor activity of the combination approach is mediated by inhibition of EGFR signaling. Taken together, these data show that treatment with a combination of lapatinib and cetuximab significantly inhibited the growth of T790M H1975 cells.

Figure 2.1. EGFR T790M mutants are resistant to gefitinib.

A, COS7 cells were transiently transfected with plasmids encoding for wild-type EGFR or for various EGFR mutants. Prior to harvesting, the cells were serum-starved for 24 hours and then grown in the presence of varying concentrations of gefitinib for 3 hours, followed by 30 minutes of EGF (100ng/mL) stimulation. Western blots are shown for phosphorylated EGFR (pY1068) and total EGFR. *B*, Western blots for phosphorylated EGFR (pY858, pY1068) and total EGFR are shown for the COS7 cell lines expressing wild-type, T790M, L858R, or L858R/T790M. *C*, Whole cell extracts were Western blotted with phosphorylated and total Stat3, Akt, and MAPK.

Figure 2.1A

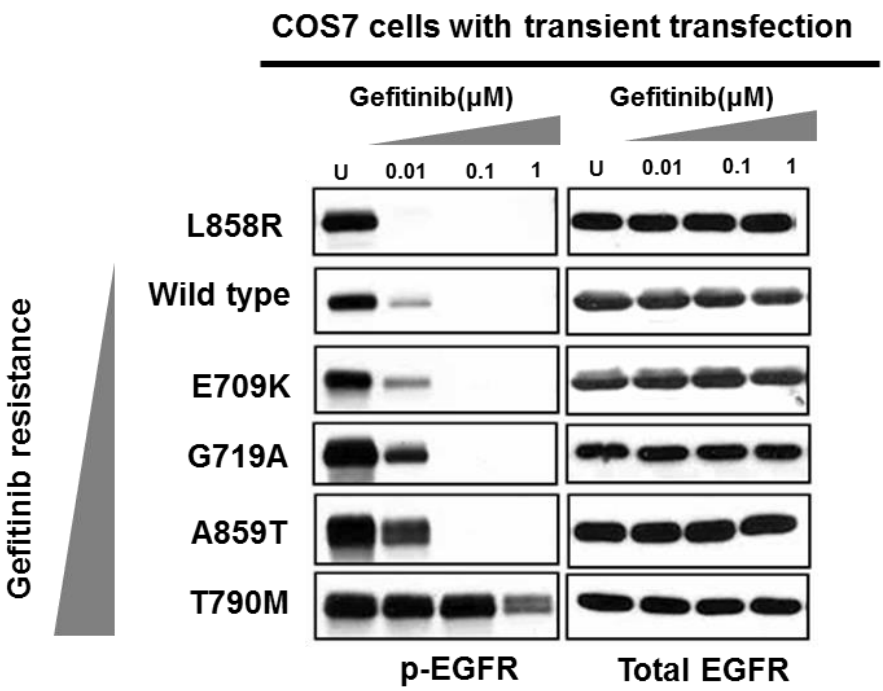

COS7 cells with transient transfection
Gefitinib(μM)
Gefitinib(μM)
U
0.01
0.1
1
U
0.01
0.1
1
L858R
Wild type
E709K
G719A
A859T
T790M
Gefitinib resistance
p-EGFR
Total EGFR

Figure 2.1B.

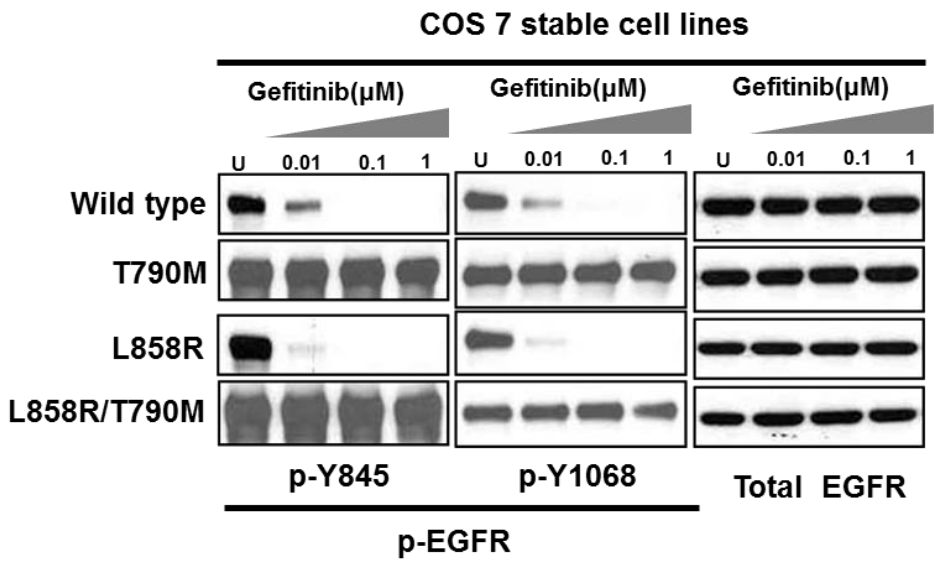

COS 7 stable cell lines
Gefitinib(μM)
Gefitinib(μM)
Gefitinib(μM)
U 0.01 0.1 1
U 0.01 0.1 1
U 0.01 0.1 1
Wild type
T790M
L858R
L858R/T790M
p-Y845
p-Y1068
Total EGFR
p-EGFR

Figure 2.1C.

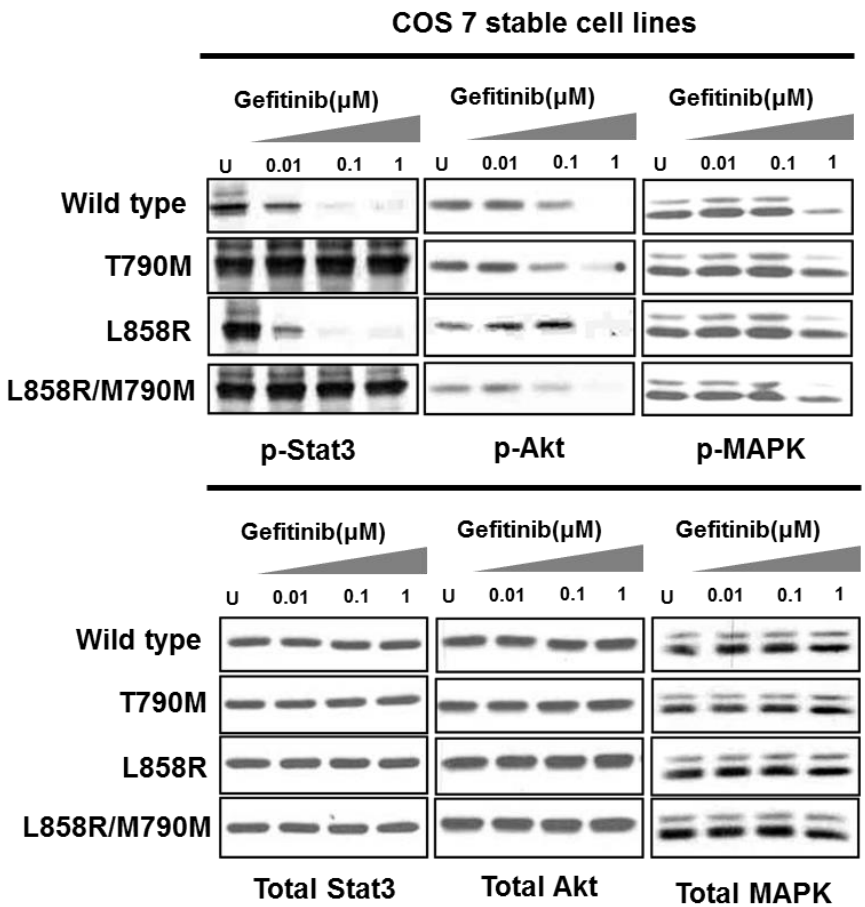

COS 7 stable cell lines
Gefitinib(μM)
Gefitinib(μM)
Gefitinib(μM)
U 0.01 0.1 1
U 0.01 0.1 1
U 0.01 0.1 1
Wild type
T790M
L858R
L858R/M790M
p-Stat3
p-Akt
p-MAPK
Gefitinib(μM)
Gefitinib(μM)
Gefitinib(μM)
U 0.01 0.1 1
U 0.01 0.1 1
U 0.01 0.1 1
Wild type
T790M
L858R
L858R/M790M
Total Stat3
Total Akt
Total MAPK

Figure 2.2. Lapatinib inhibits Stat3 and the heterodimerization of EGFR and HER2 in gefitinib-resistant T790M cells.

A, COS7-L858R/T790M cells were grown for 24 hours in the presence of cetuximab (10ng/mL), trastuzumab (10ng/mL) or lapatinib (1μmol/L). Western blots are shown for both phosphorylated and total Stat3. Values indicate the density of phosphorylated Stat3. B, H1975 cells were exposed to the indicated concentrations of gefitinib and lapatinib for 24 h. Equal amounts of total protein were then immunoprecipitated with antibodies against phosphorylated Tyr, total HER2, and EGFR. The expressed data are representative of three independent experiments. Values indicate the density of phosphorylated Tyr (Left). Whole cell extracts were Western blotted with phosphorylated and total Stat3, Akt and MAPK. C, The two EGFR L858R/T790M expressing the indicated cell lines, H1975 and COS7-L858R/T790M, were grown under the indicated conditions with gefitinib or lapatinib. After 72 hours of treatment, the cell viabilities were measured via MTT assays. The percentage of viable cells is shown relative to that of the untreated controls. The expressed data are representative of three independent experiments. D, H1975 cells were injected s.c. into nude mice

with randomization (n = 8). Once the tumors reached > 50 mm3, gefitinib (100mg/kg, p.o.) and lapatinib (100mg/kg, 150mg/kg, p.o.) were administered daily for 3 weeks. Bars, SEM repeated measures of ANOVA indicated statistically significant effects ($P<0.005$) in all groups.

Figure 2.2A.

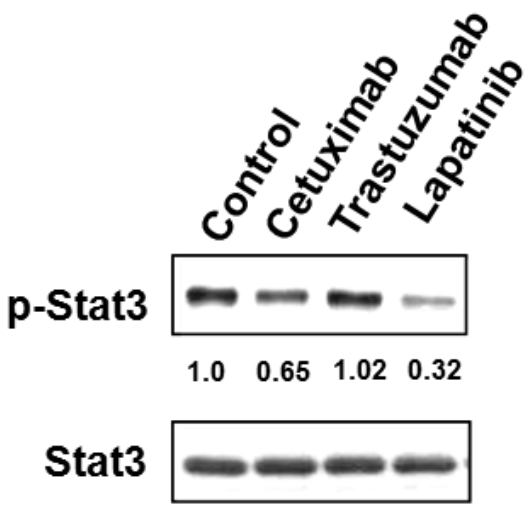

Control
Cetuximab
Trastuzumab
Lapatinib
p-Stat3
1.0
0.65
1.02
0.32
Stat3

Figure 2.2B.

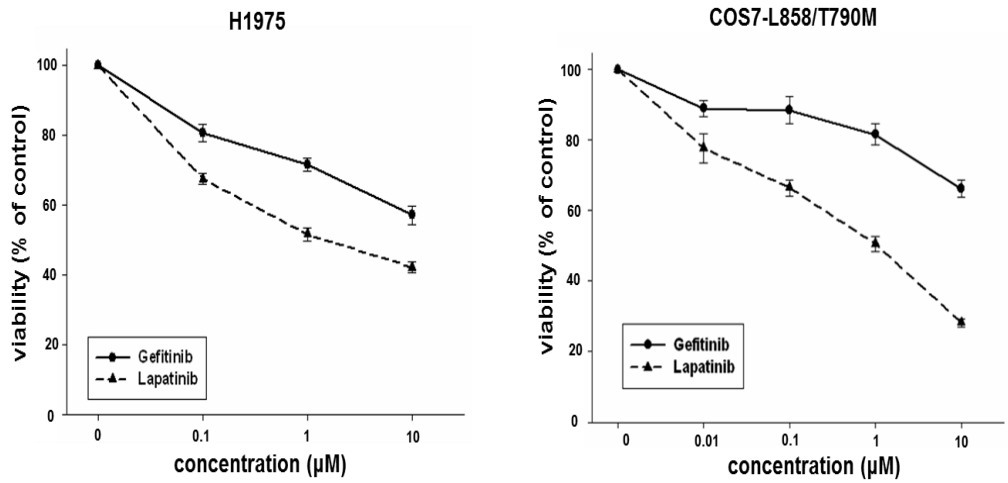

H1975
viability (% of control)
100
80
60
40
20
0
Gefitinib
Lapatinib
0
0.1
1
10
concentration (μM)
COS7-L858/T790M
viability (% of control)
100
80
60
40
20
0
Gefitinib
Lapatinib
0
0.01
0.1
1
10
concentration (μM)

Figure 2.2C.

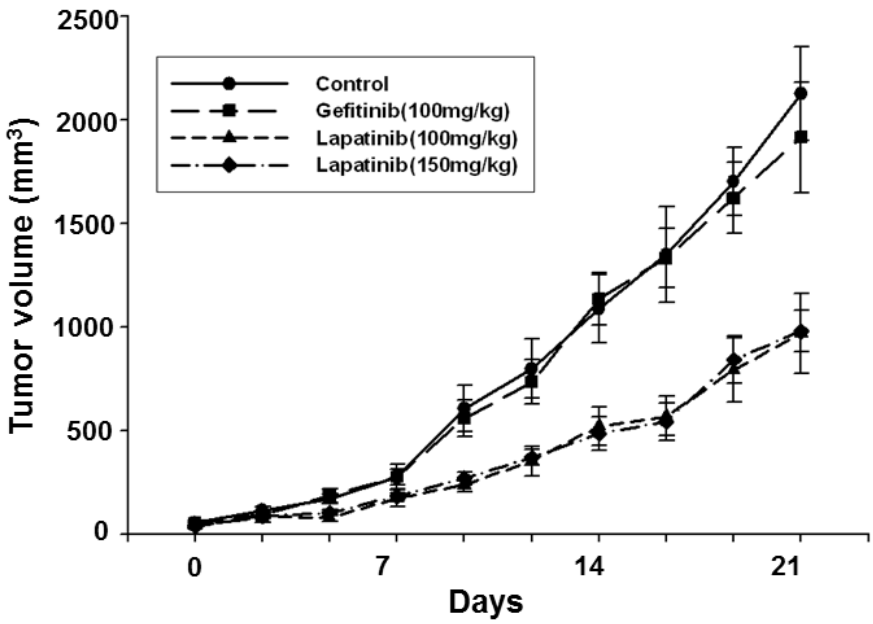

Tumor volume (mm3)
2500
2000
1500
1000
500
0
0
7
14
21
Days
Control
Gefitinib(100mg/kg)
Lapatinib(100mg/kg)
Lapatinib(150mg/kg)

Figure 2.2D.

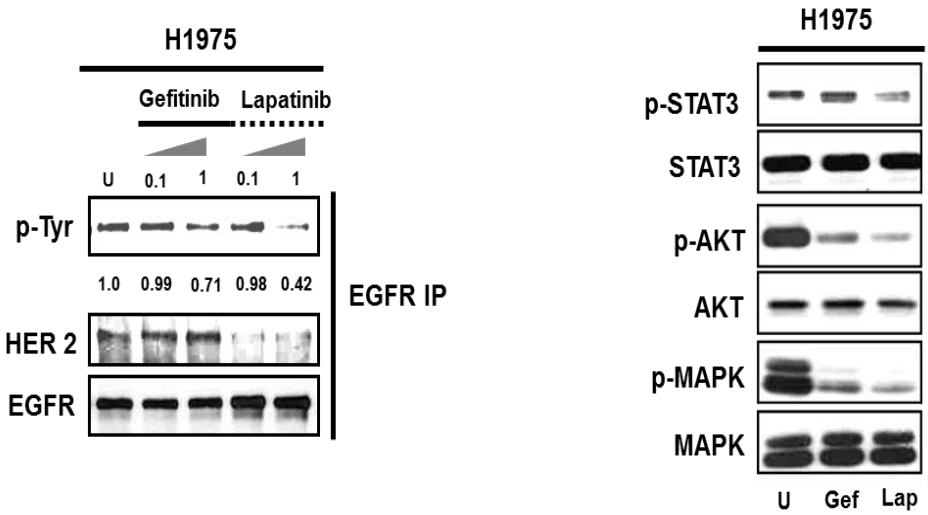

H1975
Gefitinib
Lapatinib
U
0.1
1
0.1
1
p-Tyr
1.0
0.99
0.71
0.98
0.42
EGFR IP
HER 2
EGFR
H1975
p-STAT3
STAT3
p-AKT
AKT
p-MAPK
MAPK
U
Gef
Lap

Figure 2.3. Cetuximab in combination with lapatinib enhances cytotoxicity against T790M NSCLC.

A, H1975 cells were treated with gefitinib or lapatinib with or without 0.1%, 1% and 10% serum and/or EGF (30ng/mL). After 72 hours of treatment, cell viabilities were assessed via MTT assays. B, The cells were treated with a combination of lapatinib and cetuximab (1, 10, 100ng/mL) for 72 hours, after which the cell viabilities were determined via MTT assays (Left). The cells were treated with lapatinib, cetuximab, or a combination of both at the indicated concentrations. The cells were collected, stained with Annexin V-phycoerythrin (AnnV PE) and analyzed via flow cytometry (Right). The columns show the percentage of cells staining positive for Annexin V-phycoerythrin; Columns, means; bars, ±SD. * and **, $P<0.05$, combination versus singles. C, Cells were treated with lapatinib (1μmol/L), cetuximab (10ng/ml), or a combination of both for 72 hours. Western blots are shown for the indicated proteins. D, H1975 cells were injected s.c. into nude mice with randomization (n = 8). Treatment with lapatinib (100mg/kg, p.o., daily for 3 weeks) and cetuximab (10mg/kg, i.p., twice weekly for 3 weeks) was initiated once the tumors achieved a volume of 50 mm3. Bars, SEM repeated measures of ANOVA indicated statistically significant effects ($P<0.005$) in all

groups. Tumor samples treated with lapatinib, cetuximab, or a combination thereof were collected at the end point and homogenized. Western blots are shown for phosphorylated and total EGFR. -tubulin was employed as a loading control.

Figure 2.3A.

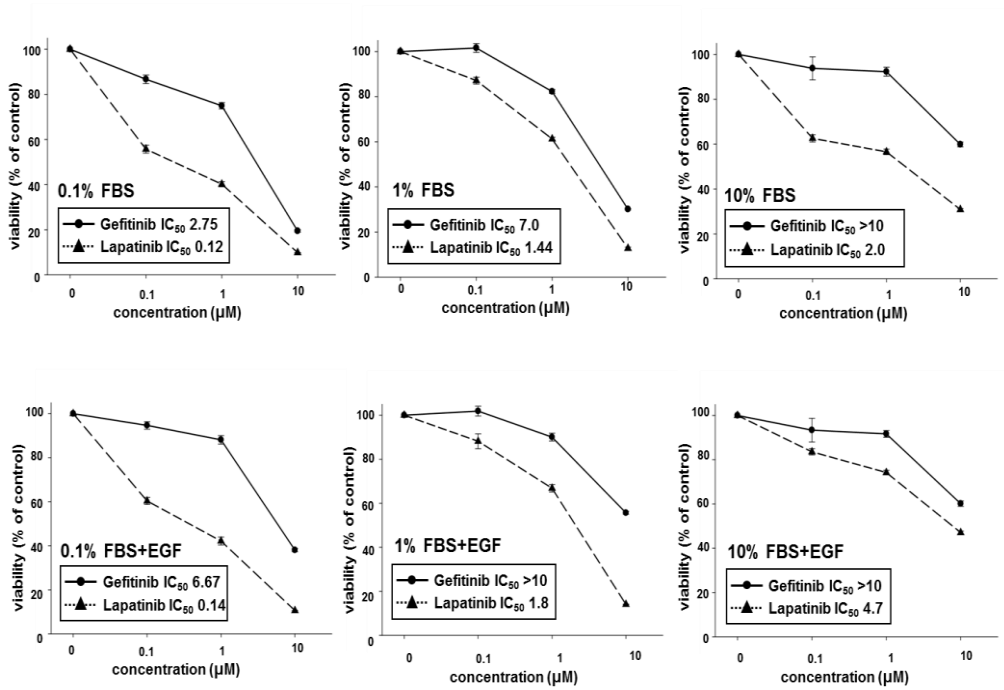

viability (% of control)
concentration (μM)
0.1% FBS
Gefitinib IC50 2.75
Lapatinib IC50 0.12
1% FBS
Gefitinib IC50 7.0
Lapatinib IC50 1.44
10% FBS
Gefitinib IC50 >10
Lapatinib IC50 2.0
0.1% FBS+EGF
Gefitinib IC50 6.67
Lapatinib IC50 0.14
1% FBS+EGF
Gefitinib IC50 >10
Lapatinib IC50 1.8
10% FBS+EGF
Gefitinib IC50 >10
Lapatinib IC50 4.7

Figure 2.3B

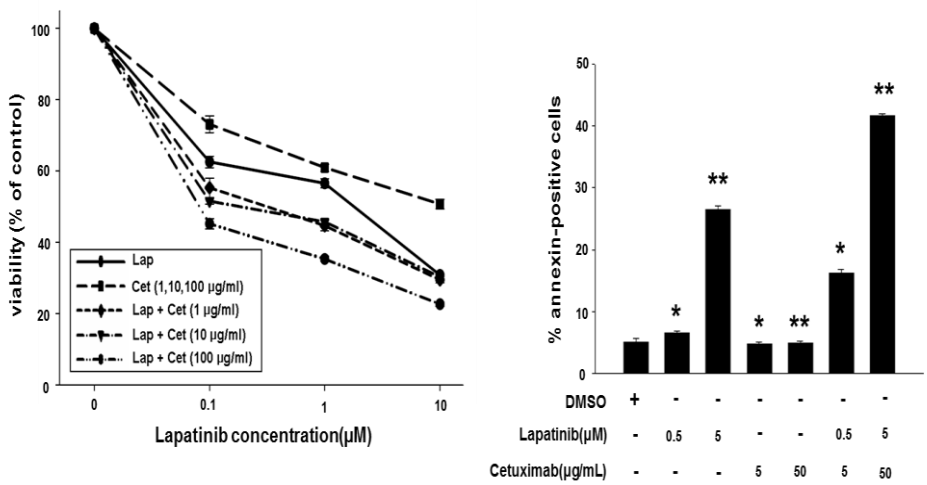

viability (% of control)
100
80
60
40
20
0
0
0.1
1
10
Lapatinib concentration(μM)
Lap
Cet (1,10,100 μg/ml)
Lap + Cet (1 μg/ml)
Lap + Cet (10 μg/ml)
Lap + Cet (100 μg/ml)
% annexin-positive cells
50
40
30
20
10
0
*
**
*
**
*
**
DMSO + - - - - - -
Lapatinib(μM) - 0.5 5 - - 0.5 5
Cetuximab(μg/mL) - - - 5 50 5 50

Figure 2.3C.

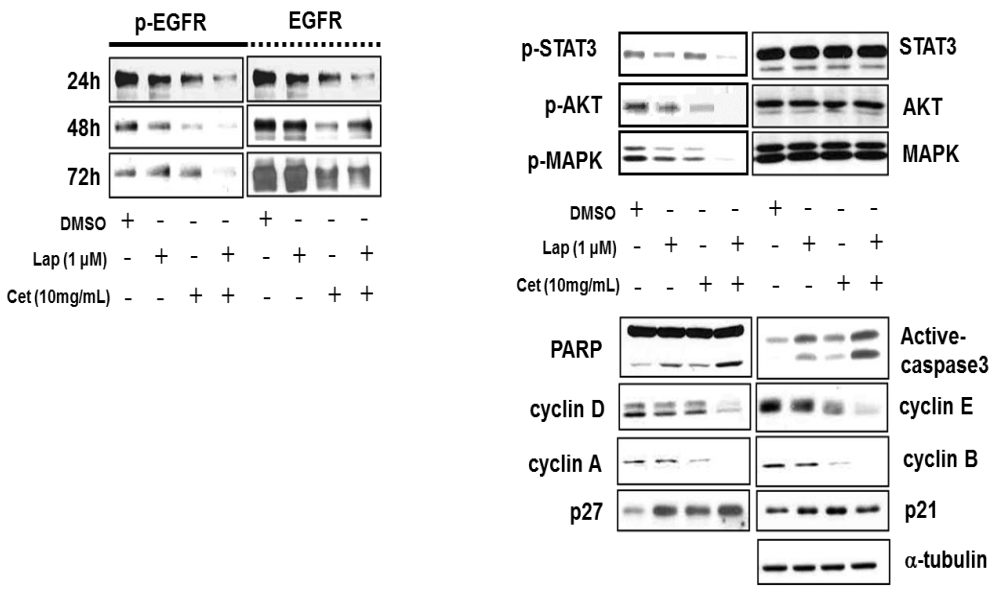

p-EGFR
EGFR
24h
48h
72h
DMSO + - - - + - - -
Lap (1 μM) - + - + - + - +
Cet (10mg/mL) - - + + - - + +
p-STAT3
STAT3
p-AKT
AKT
p-MAPK
MAPK
DMSO + - - - + - - -
Lap (1 μM) - + - + - + - +
Cet (10mg/mL) - - + + - - + +
PARP
Active-caspase3
cyclin D
cyclin E
cyclin A
cyclin B
p27
p21
α-tubulin

Figure 2.3D.

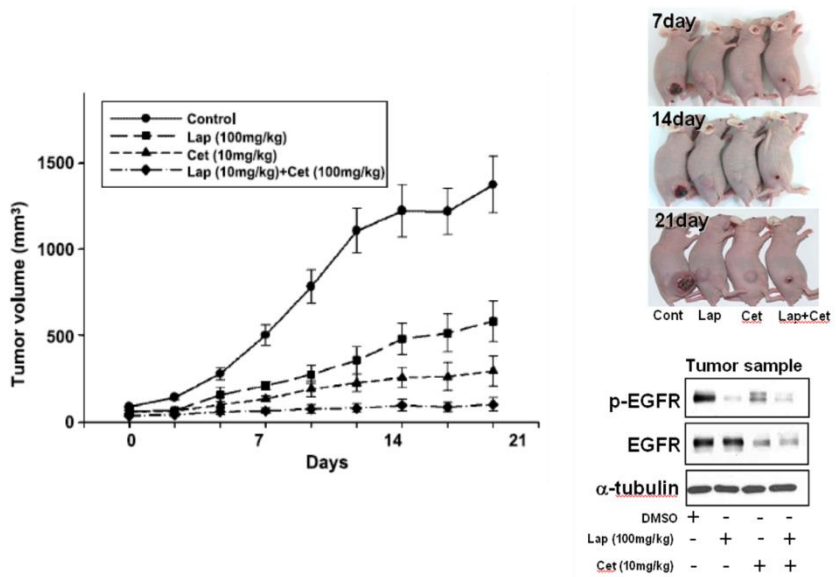
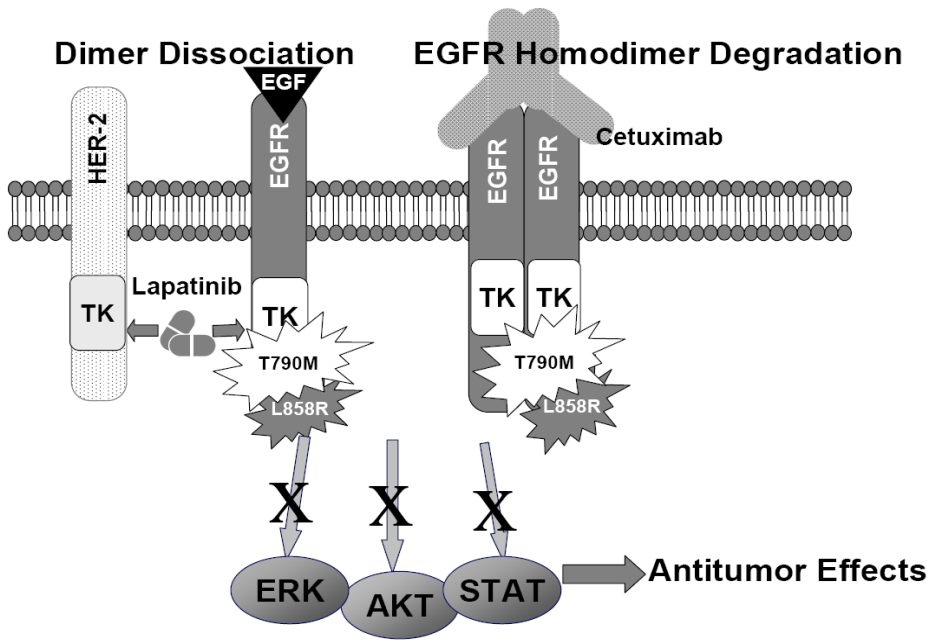

Control
Lap (100mg/kg)
Cet (10mg/kg)
Lap (10mg/kg)+Cet (100mg/kg)
Tumor volume (mm³)
1500
1000
500
0
0
7
14
21
Days
7day
14day
21day
Cont
Lap
Cet
Lap+Cet
Tumor sample
p-EGFR
EGFR
α-tubulin
DMSO + - - -
Lap (100mg/kg) - + - +
Cet (10mg/kg) - - + +

Figure 2.4. Proposed model showing the effects a lapatinib and cetuximab combination treatment in gefitinib-resistant T790M cells. T790M lung cancer cells were resistant to gefitinib, and Stat3 was persistently activated in these cells. Lapatinib reduced Stat3 activation by blocking the heterodimerization of EGFR and HER2, and cetuximab effected a complete downregulation of EGFR. Therefore, the combination of lapatinib and cetuximab, which induces dimeric dissociation and EGFR downregulation, results in antitumor effects on T790M cells.

Figure 2.4.


Dimer Dissociation
EGFR Homodimer Degradation
EGF
HER-2
EGFR
EGFR
EGFR
Cetuximab
TK
Lapatinib
TK
TK
TK
T790M
T790M
L858R
L858R
X
X
X
ERK
AKT
STAT
Antitumor Effects

DISCUSSION

Understanding genetic alterations of tumors is essential for the successful development of novel strategies to circumvent resistance to TKIs. Currently, it is known that irreversible EGFR TKIs, such as HKI-272 and HKI-357, may overcome gefitinib resistance in T790M cells by irreversibly binding to the ATP binding cleft, even in the presence of the T790M mutation. Although irreversible binding capacity of those TKIs appears to be the main reason for overcoming gefitinib resistance, it should also be noted that these TKIs target not only EGFR, but also HER2. Downregulation of HER2 causes a loss of viability in T790M cells, suggesting the importance of HER2 inhibition in overcoming resistance to EGFR TKI. Similar to these results, our study demonstrated that lapatinib dissociated heterodimerization of EGFR and HER2 in T790M cells, whereas geftinib-resistant T790M cells maintained EGFR/HER2 heterodimerization in spite of gefitinib treatment. Lapatinib is a reversible dual TKI for both EGFR and HER2 that binds to an inactive conformation of EGFR that is quite different from the active structure bound by the selective EGFR inhibitor, erlotinib, which suggests that this TKI has a different mechanism of actions [59]. Therefore, besides irreversibility of TKIs,

our data also suggest that inhibition of HER2 may play a role in reversing the resistance in terms of receptor dedimerization. Moreover, lapatinib has a very slow off-rate from purified interacellular domains of EGFR and HER2 compared with erlotinib or gefitinib. Lapatinib is currently under active clinical investigation and has shown promising activity against HER2 overexpressing breast cancer cells. However, the efficacy of lapatinib has not been investigated in NSCLC, and its role in EGFR TKI-resistant NSCLC is unknown. In this study, we demonstrated that lapatinib revealed moderately enhanced cytotoxicity against gefitinib-resistant T790M cells in vitro and in vivo. Based on its de-dimerization effect on EGFR and HER2, lapatinib may partially attenuate the resistance to EGFR TKI in T790M lung cancer cells.

EGFR mutant cells are constitutively activated by autophosphorylation of mutant TK, thereby transducing proliferation signals to cancer cells [60, 61] . These signals are the therapeutic basis of the use of TKI for EGFR mutant cells, since the growth of EGFR mutant cells are primarily dependent on TK activity regardless of ligands binding. In particular, it is previously reported that T790M mutant cells are totally independent of ligand binding, which means that the growth of T790M cells are primarily mediated by autophosphorylated TK . However, in this study, I observed that the presence of serum or EGF

reduced the antiproliferative effect of lapatinib in T790M cells, which suggests that these cells may retain ligand-dependent growth in part and thus cetuximab's competition for the binding of ligands may be responsible for its effectiveness in combination therapy. Ceuximab's antitumor mechanisms include inhibition of ligands binding, receptor internalization, and degradation or down-regulation of the receptor. However, there is a controversy about the efficacy of cetuximab in EGFR mutant cells. Initial experiments have shown that cetuximab is less effective than gefitinib in the treatment of EGFR mutants [62, 63]. Conversely, recent studies have shown that cetuximab inhibited the tumor growth of L858R/T790M H1975 cells by downregulating EGFR. In this study, I also found that cetuximab downregulated mutant EGFR in T790M cells, and furthermore treatment with a combination of cetuximab and lapatinib significantly inhibited tumor growth both in vitro and in vivo (Figure 2.3B-2.3D). Various biochemical studies and apoptotic assay have shown the synergistic activity of combined lapatinib and cetuximab treatment in gefitinib-resistant T790M cells. Taken together, I propose that combination of lapatinib and cetuximab is mechanistically co-operative since lapatinib affects receptor heterodimerization and cetuximab primarily reduces the level of EGFR proteins (Figure. 2.4). In gefitinib-resistant T790M lung cancer cells,

lapatinib reduced Stat3 activation by blocking the heterodimerization of EGFR and HER2, and cetuximab effected a complete downregulation of EGFR. The combination of lapatinib and cetuximab completely blocked the activation of downstream signals including Stat3, resulting in enhanced cytotoxicity against in T790M cells. Therefore, this combination provides a therapeutic concept of a complete blockade of EGFR signaling for cancer therapy. Because both drugs are currently being used in clinics, these findings can be directly tested in future clinical investigations. Our current observations also raise important clinical issues. First, the combination of lapatinib and cetuximab needs to be investigated in NSCLC patients whose disease has progressed after treatment with gefitinib. Second, this combination should also be tested to determine whether lapatinib is superior to other EGFR TKIs when combined with cetuximab.

In conclusion, these data suggest that treatment with a combination of lapatinib and cetuximab is promising for the management of NSCLC patients that exhibit resistance to EGFR TKI.

Ⅳ. Testican-1 mediated epithelial-mesenchymal transition signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer

ABSTRACT

Human epidermal growth factor receptor 2 (HER2)-directed treatment using trastuzumab has shown clinical benefit in HER2-positive gastric cancer. Clinical trials using lapatinib in HER2-positive gastric cancer are also currently underway. As with other molecularly targeted agents, the emergence of acquired resistance to HER2-directed treatment is an imminent therapeutic problem for HER2-positive gastric cancer. In order to investigate the mechanisms of acquired resistance to HER2-directed treatment in gastric cancer, I generated lapatinib-resistant gastric cancer cell lines (SNU216 LR) in vitro by chronic exposure of a HER2-positive gastric cancer cell line (SNU216) to lapatinib. The resultant SNU216 LR cells were also resistant to gefitinib, cetuximab, trastuzumab, afatinib and dacomitinib. Interestingly, SNU216 LR cells displayed an epithelial-mesenchymal transition (EMT) phenotype and maintained the activation of MET, HER3, Stat3, Akt and mitogen-activated protein kinase signaling in the presence of lapatinib. Using gene expression arrays, I identified the upregulation of a variety of EMT-related genes and extracellular matrix molecules, such as Testican-1, in SNU216 LR cells. I showed that the inhibition of Testican-1 by small

interfering RNA decreased Testican-1-induced, MET-dependent, downstream signaling, and restored sensitivity to lapatinib in these cells. Furthermore, treatment with XAV939 selectively inhibited β-catenin-mediated transcription and Testican-1-induced EMT signaling, leading to G1 arrest. Taken together, these data support the potential role of EMT in acquired resistance to HER2-directed treatment in HER2-positive gastric cancer, and provide insights into strategies for preventing and/or overcoming this resistance in patients

INTRODUCTION

Overexpression of HER2 in human gastric cancer, particularly in the advanced stages of disease, correlates with aggressive tumor growth and poor prognosis. Moreover, HER2 positivity was shown to be higher in intestinal compared with diffuse types of cancer. Thus, HER2 is emerging as an important therapeutic target in gastric cancer [64-66]. Recently, ToGA clinical trial comparing the effect of combined chemotherapy and trastuzumab versus chemotherapy alone in patients with HER2-positive, advanced gastric cancer, showed clinical benefits in terms of overall survival, progression-free survival and response rate [67]. The antitumor activity of lapatinib, an EGFR and HER2 dual tyrosine kinase inhibitor (TKI), has also been examined in gastric cancer cells [33, 68]. Currently, a phase III clinical trial comparing the effect of combined lapatinib and chemotherapy versus chemotherapy alone in HER2-positive patients with gastric cancer is ongoing. Although overexpression of HER2 correlates with sensitivity to growth inhibition by lapatinib, not all cells overexpressing HER2 respond to lapatinib [69], and resistance has been observed in a subset of patients following chronic exposure. The factors that confer primary or acquired resistance to lapatinib

are not well characterized.

Recent studies have shown that resistance to EGFR-TKI in non-small cell lung cancer (NSCLC) cell lines, correlates with epithelial-mesenchymal transition (EMT) that affects cell-cell contacts [70-75]. EMT is a unique process whereby epithelial cells undergo remarkable morphologic changes, characterized by a transition from an epithelial cobblestone to an elongated mesenchymal phenotype, leading to increased motility and invasion. EMT progression is characterized by the loss of proteins involved in cell junctions, such as E-cadherin, and the expression of mesenchymal markers such as Vimentin and Snail. NSCLC cells acquiring a mesenchymal phenotype may therefore be resistant to TKI inhibition, since mesenchymal-like cells are capable of bypassing EGFR signaling, acquiring alternative routes of proliferative and survival signaling. For this to occur, extracellular matrix (ECM) molecules or membrane receptors must cooperate to specify these alternative signaling pathway(s) or to allow synergistic or antagonistic relationships, facilitating the communication of cancer cells with diverse extracellular cues. EMT is a dynamic process triggered by the interplay of several extracellular signals, including collagen. Many signaling pathways, including the Wnt/ -catenin, transforming growth factor (TGF)-β, Hedgehog and integrin pathways

are critical for EMT induction [76, 77]. However, the exact mechanism underlying the acquisition of the EMT phenotype in EGFR-TKI-resistant lung cancer cells remains unknown.

Testican-1 was first defined as an unnamed chondroitin/heparan sulfate proteoglycan in seminal plasma [78]. Testican-1 is a component of the ECM, with predicted roles in cell adhesion and migration. Initially, it was thought that Testicans may contribute to the proteoglycan-rich ECM of the brain and play a role neurogenesis. Furthermore, overexpression of Testican has been reported in in prostate, pancreatic and hepatocellular carcinoma, in addition to gastric neuroendocrine tumors [79-81]. However, the biological function of Testicans has not been extensively explored in cancer. I recently demonstrated that Testican-1 induced EMT signaling confers resistance to lapatinib.

In this study, I established lapatinib-resistant cell lines from HER2-positive, lapatinib-sensitive, SNU216 human gastric cancer cells. I demonstrate that increased Testican-1 induces EMT, representing a novel mechanism of resistance to lapatinib and trastuzumab. Inhibition of EMT by XAV939 selectively inhibited β-catenin-mediated transcription, restoring lapatinib sensitivity to these cells. Testican-1 deprivation or inhibition of the

β-catenin pathway, restored lapatinib sensitivity by down-regulating EMT signaling. These data support the development of novel, therapeutic approaches for treating HER2-positive gastric cancer. Furthermore, these data support a potential role of EMT transition in acquired resistance to HER2-directed treatment in HER2-positive gastric cancer, providing unique insights into strategies for preventing and overcoming disease resistance.

MATERIAL AND METHODS

1. Cell culture and reagents

The SNU216 human gastric cancer cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 media containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37 °C under 5% CO2. Lapatinib was a gift from GlaxoSmithKline (Research Triangle Park, NC). Gefitinib, Afatinib, Dacomitinib and XAV-939 was purchased from Selleck Chemicals (Houston, TX). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C .

2. Generation of lapatinib-resistant clones from SNU 216 cells in vitro

SNU216 cells were exposed to increasing concentrations of lapatinib over a period of 8 months, reaching a final concentration of 10 μmol/L at the end of this period. Single cell clonal populations were obtained by serial dilution or isolation with cloning cylinders. Cells were expanded in RPMI-1640 medium containing 10% FBS containing lapatinib (1 μmol/L).

3. Growth inhibition Assays

The viability of cells was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays (Sigma-Aldrich, St. Louis, MO). A total of 3 × 103 cells were seeded in 96-well plates, incubated for 24 h, and treated for 72 h with indicated drugs at 37 °C. Following treatment, MTT solution was added to each well and incubated for 4 h at 37 °C. Medium was then removed, and DMSO was added and mixed thoroughly for 30 min at room temperature. Cell viability was determined by measuring absorbance at 540 nm using a VersaMax™ microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of drug required to inhibit cell growth by 50% was determined via interpolation from dose-response curves using Calcusyn software (Biosoft, Ferguson, MO). Six replicate wells were utilized for each analysis, and at least three independent experiments were conducted. The data from replicate wells are presented as the mean number of the remaining cells with 95% confidence intervals (CI).

4. Western blot and phospho-RTK Arrays

Antibodies against p-EGFR (pY1068), p-HER2(pY1221/1222), p-

HER3(pY1289), p-Stat3 (pY705), p-Akt (pS473), p-MAPK (Thr202/Tyr204), EGFR, HER2, HER3, Akt , MAPK, vimentin, E-cadherin and Axin were purchased from Cell Signaling Technology (Beverley, MA). Anti-β- catenin, cyclin D and p27 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Sub-confluent cells (70-80%) were used for protein analyses. For the analysis of signaling post-drug treatments, cells were treated with different concentrations of lapatinib or XAV-939 for 48 h and lysed in RIPA or NP40 buffer. Immunoprecipitation was performed as previously described and analyzed by western blot using an enhanced chemiluminescence system. Phospho-RTK arrays were purchased from R&D Systems and were conducted in accordance with the manufacturer's instructions.

5. Short Interfering RNA (siRNA) Knockdown

siRNA against Testican-1 was purchased from mbiotech (Seoul, Korea). Cells were transfected with siRNAs (40 nmol/L) using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Cell lysates were harvested 48 h after transfection.

6. Microarray analysis

Total RNA was extracted from SNU216 and SNU216-LR4 cells using an RNeasy mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. RNA (xug) was hybridized on HG-U133 Affymetrix Genechip arrays (Affymetrix, Santa Clara, CA) by an Agilent certified service provider (DNA Link, Seoul, South Korea). All samples were analyzed and reported according to MIAME guidelines. Gene expression changes in SNU216-LR4 cells compared with the SNU216 cells (≥ 2 fold) were identified using the GeneExpress Software System Fold Change Analysis tool. For each gene fragment, the ratio of the geometric means of the expression intensities in SNU216 and SNU216-LR4 cells was calculated, and the fold change was then calculated on a per fragment basis. Confidence limits were calculated using a two-sided Welch modified t-test for the difference of the means for the logs of the intensities.

7. Reverse Transcription-PCR and Real-Time PCR Analysis

Real-time reverse-transcription polymerase chain reaction (RT-PCR) Total RNA was extracted with TRI reagent (Molecular Research Center, Cincinnati,

OH), and cDNA was synthesized from 1 μg of total RNA with ImPrm-IITM reverse transcriptase (Promega Corporation, Madison, WI) using random hexamers. RT-PCR was performed using SYBR green I (Molecular Probe, Eugene, OR) and an iCycler IQ detection system (Bio-Rad Laboratories, Hercules, CA). All reactions were performed in triplicate. The primers used for RT-PCR are as follows: Testican-1, forward primer 5′-GCAAATTGGAGTTCCATGCT-3′ and reverse primer 5′-TCCTTTCTGCCTTGTGCTTT-3′, Vimentin, forward primer 5′-CCTCACCTGTGAAGTGGATGC-3′ and reverse primer 5′-CAACGGCAAAGTTCTCTTCCA-3′, Twist1, forward primer 5′-TGTCCGCGTCCCACTAGC-3′ and reverse primer 5′-TGTCCATTTTCTCCTTCTCTGGA-3′, Snail1, forward primer 5′-TGCAGGACTCTAATCCAAGTTTACC-3′ and reverse primer 5′-GTGGGATGGCTGCCAGC-3′, Actin, forward primer 5′-AGAGCTACGAGCTGCCTGAC-3′ and reverse primer 5′-GGATGCCACAGGACTCCA -3′.

7. Wound-healing assay

Cells were seeded at a high density on 60-mm culture dishes. Twelve hours later, wounds were made by scraping through the cell monolayer with a pipette tip. After washing, the cells were incubated in growth medium for 24 h and observed under a microscope. The wound closure was estimated as the ratio of the remaining wound area relative to the initial wounded area.

8. Cell cycle analysis

Cells were washed twice in phosphate buffered saline (PBS), fixed in 70% ethanol and incubated at -20 °C, until analysis was performed. Prior to analysis, cell suspensions were washed with PBS, digested with RNase A (50 µg/ml) for 15 min at 37 °C and stained with propidium iodide (50 µg/ml). DNA content (10,000 cells/experimental group) was determined using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) equipped with a ModFit LT program (Verity Software House Inc.), as previously described [68].

9. Statistical analysis

All experiments were conducted in duplicate or triplicate, with at least two biological replicates. All data are expressed as the mean ± SD. Statistical significance was calculated using an unpaired Student's t-test, and values of $p < 0.05$ were considered statistically significant.

RESULTS

1. Activation of c-MET and HER3 confers acquired resistance to lapatinib-sensitive HER2-positive gastric cancer cells

To explore the mechanisms of lapatinib resistance, I generated lapatinib-resistant gastric cancer cell lines using HER2-positive, lapatinib-hypersensitive SNU216 cells. Cells were exposed to increasing concentrations of lapatinib for 8 months, and single cell clones were isolated from a pool of resistant cells (SNU216 LR)(full clones) to generate the resultant cell lines, SNU216 LR4-LR10 (7 clones), which displayed resistance to lapatinib in vitro (IC50 > 10 μmol/L; Figure 3.1A). The phosphorylation status of HER family kinases and downstream targets of Stat, PI3K/Akt and MEK signaling were determined for SNU216 and SNU216 LR cell lines. Treatment of parental SNU216 cells with lapatinib markedly inhibited tyrosine phosphorylation of EGFR, HER2 and HER3 in addition to Stat3, Akt and mitogen-activated protein kinase (MAPK). In contrast, phosphorylation of these proteins persisted at higher levels in lapatinib-resistant SNU216 LR cell lines following treatment (Figure 3.1B). I next investigated whether aberrant

activation of other receptors may play a role in mediating lapatinib resistance. I performed a phospho-receptor tyrosine kinase (phospho-RTK) array to compare the effects of lapatinib on phosphorylation of 42 RTKs in SNU216 and SNU216 LR cells (full clones) (Figure 3.1C). In SNU216 LR cells, EGFR, HER2, HER3 and MET were all phosphorylated, and phosphorylation persisted following lapatinib treatment, compared with parental cells. These results were confirmed using additional resistant clones (LR4, LR6 and LR7). Furthermore, I also observed that total levels of HER3 and MET were elevated in resistant clones (Figure 3.1D). Previous studies have shown that acquired resistance to EGFR TKIs, such as erlotinib or gefitinib, is associated with a secondary mutation or MET amplification in NSCLC (91). Analysis of MET copy number in SNU216 LR lapatinib-resistant cells revealed no amplification compared with parental SNU216 cells. Furthermore, genomic DNA sequence analysis of the HER2 kinase domain in SNU216 and SNU216 LR cell lines revealed no mutations (data not shown). Taken together, these data suggest that activation of c-MET and HER3 confers acquired resistance to lapatinib-sensitive, HER2-positive gastric cancer cells via persistent activation of Stat3, Akt and MAPK, the main downstream signaling effectors of EGFR and HER2.

2. Lapatinib-resistant cell lines are resistant to growth inhibition by HER-targeted inhibitors

Next, I used MTT assays to assess the ability of HER-targeted inhibitors, gefitinib (an EGFR TKI), afatinib (an irreversible EGFR and HER2 TKI), dacomitinib (a pan-HER TKI), cetuximab (an EGFR monoclonal antibody) and trastuzumab (a HER2 monoclonal antibody), to inhibit the growth of lapatinib-resistant clones (Figure 3.2A). I previously reported that these HER-targeted inhibitors (except cetuximab), are active in HER2-positive SNU216 gastric cancer cells [82, 83]. Half-maximum inhibitory concentrations (IC_{50}) of gefitinib, afatinib, dacomitinib on lapatinib resistant cell lines (LR4, LR5 and LR6) were 500-fold higher against the parental cells. Moreover, the lapatinib-resistant clones were also resistant to growth inhibition by trastuzumab, compared with parental cells. In contrast, the inhibitory effect of cetuximab was similar in both parental and resistant clones. To further confirm these observations, I next assessed the levels of total and phosphorylated Stat3, Akt and MAPK (Figure 3.2B). I observed that the phosphorylation status of Stat3, Akt and MAPK tended to be higher in SNU216 LR4 cells compared with parental cells. However, no significant differences in Stat3, Akt and MAPK phosphorylation were observed between SNU216 cells and SNU216 LR4 clones, following

treatment with HER-targeted inhibitors . These results were confirmed in all lapatinib-resistant cell lines (data not shown). Moreover, levels of phosphorylated Akt and MAPK were not decreased in all lapatinib-resistant cell lines following trastuzumab treatment (Figure 3.2C). Taken together, these results demonstrate that lapatinib-resistant cells are also resistant to HER-targeted inhibitors, including trastuzumab.

3. Lapatinib-resistant cells exhibit upregulation of EMT-associated gene signatures

To identify genes that may play a role in acquired resistance to lapatinib, I investigated genes differentially expressed between sensitive (parental) and resistant (LR4) cell lines using the class comparison tool in cDNA microarrays. Clustering Analysis of 205differentially expressed genes (Supplementary Table S1, $P < 0.005$) identified enrichment of several, interesting networks/pathways including those involved in cell cycle, regulation of apoptosis, focal adhesion, ECM-receptor interaction, immune response, leukocyte activation, antigen progression and presentation and leukocyte migration. However, one of the most striking patterns emerging from these

analyses involved genes implicated in EMT, including genes required for extra-cellular matrix (Testican-1 and Timp3), EMT (TGF-β, Smad3, Tgm2, Twist1 and Foxm1), migration (COL18A1, ITGA6, FAS and FYN), and cell-cycle associated genes (Figure 3.3A). All genes in Figure 11A had statistical significance at the cut off of $P < 0.005$. Differential expression of EMT-associated genes (Vimentin, Twist1, Snail and β-catenin) and Testican-1, which was highly differentially expressed, was confirmed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using several resistant clones (Figure 3.3B). I next analyzed protein levels of the epithelial marker, E-cadherin, and the mesenchymal marker, vimentin, in three resistant clones. These analyses revealed that parental cell lines expressed E-cadherin, whereas resistant clones expressed high levels of vimentin, confirming the gene expression and RT-PCR results. I also confirmed the increase in Testican-1 and β -catenin at the protein level, in LR cell lines (LR4, LR6, LR7; Figure 3.3C). Interestingly, β-catenin levels were increased in LR4 cells, even though this is an epithelial marker. Furthermore, the LR cells displayed elongated, irregular fibroblastoid morphology. In contrast, parental cells displayed a rounded shape, typical of an epithelial cobblestone appearance, and these cells grew in clusters. These morphological changes suggest that

LR cells have undergone EMT, as reported previously [73]. To further confirm the EMT of LR cells, I also investigated cell migration. As I expected, parental cells were less migratory than LR cells (Figure 3.3D). Our results clearly demonstrate upregulation of EMT signaling in lapatinib-resistant cells. Taken together, these data indicate that EMT signaling may play a crucial role in lapatinib resistance in this cell model.

4. Lapatinib resistance is reduced via suppression of Testican-1

To determine whether Testican-1-induced EMT signaling underlies acquired resistance to lapatinib, I examined whether inhibition of Testican-1 could suppress growth of resistant cells. Cell viability was measured in LR4 and LR7 cells, following siRNA-mediated knockdown of Testican-1 and treatment with lapatinib. Treatment with lapatinib (1 μmol/L) alone or targeted silencing of Testican-1 had no effect in either LR4 or LR7 cells. However, in the presence of lapatinib treatment, knockdown of Testican-1 suppressed cell growth (~60%) compared with si-control in both cell lines (Figure 3.4A). To confirm this, I treated Testican-1 overexpressing SNU216 cells with lapatinib and analyzed cell viability. In contrast to SNU216 mock-infected cells, which

exhibited growth inhibition following lapatinib treatment (1 μmol/L), inhibition was not observed in cells overexpressing Testican-1 (Figure 3.4B). To further confirm this result, I performed western blot analyses to determine total and phosphorylated levels of MET, HER3, Vimentin and β-catenin. Knockdown of Testican-1 led to a decrease in phosphorylated MET and HER3. Furthermore, the levels of Vimentin and β-catenin were significantly decreased (Figure 3.4C). These results suggest that Testican-1 plays a direct role in lapatinib resistance in HER2-positive gastric cancer cells.

5. Inhibition of WNT/β-catenin signaling restores sensitivity to lapatinib

Previously, I showed that silencing of Testican-1 inhibits activated MET and HER3, and also leads to a decrease in the EMT regulators, Vimentin and β-catenin. WNT/β-catenin signaling is involved in EMT induction during tumor progression and converts polarized epithelial cells into motile, invasive cells [84, 85]. Based on this, I reasoned that inhibition of WNT/β-catenin may restore lapatinib sensitivity caused by Testican-1 induced EMT signaling. To test this hypothesis, I investigated the effect of combined treatment with lapatinib and XAV939 on the growth of LR cells. XAV939 is a small molecule

inhibitor for the WNT/ β -catenin pathway [86]. As shown in Figure. 3.5A and B, treatment of cells with this drug combination had a greater inhibitory effect on cell growth compared with treatment with single-agent XAV939 in LR4 cells. XAV939 enhanced the inhibitory effect of lapatinib on cell growth in a dose-responsive manner. I next investigated cell cycle, following staining of cells with the DNA-intercalating dye, PI. The sensitivity of LR cells to lapatinib may result from cell cycle arrest at specific phases. As shown in Figure 3.5C, no significant change in cell cycle distribution was detected in cells treated with XAV939 alone. However, XAV939 increased the proportion of cells in the G1 phase of lapatinib-treated cells. I next examined the levels of total and phosphorylated Stat3, Akt, MAPK, β-catenin, cyclin D and p27 in LR4 cells (Figure 3.5D). Treatment with lapatinib or XAV939 alone had no effect of inhibition detected to inhibit a phosphorylation of Akt and MAPK with decrease of the expression of cell cycle regulatory molecules examined. However, combined treatment with XAV939 and lapatinib led to a significant decrease in phosphorylated Stat3, Akt and MAPK. Cyclin D was also decreased after treatment with combination therapy, whereas the cell cycle inhibitor, p27 was increased. Taken together, these data show that combined treatment with lapatinib and XAV939 significantly inhibits the growth of lapatinib-resistant

SNU216 LR4 cells.

Figure 3.1. SNU216 LR cells are resistant to lapatinib in vitro and display activation of c-MET and HER3.

A. Lapatinib resistant sub-lines of the HER2-positive, SNU216 human gastric cancer cell line were generated by culturing in increasing concentrations of lapatinib over an 8-month period. SNU216 and eight resistant (SNU216 LR4-LR10) cell lines were treated with lapatinib at the indicated concentrations and cell viability was measured after 72 h. The percentage of viable cells is shown relative to untreated controls. B. SNU216 and SNU216-LR cells (full clones) were exposed to increasing concentrations of lapatinib (0.01, 0.1 and 1 μmol/L) for 24 h. Whole-cell extracts were analyzed by western blot with antibodies recognizing phosphorylated and total EGFR, HER2, HER3, Stat3, Akt and MAPK. C. SNU216 and SNU216 LR cells (full clones) were treated with 1 μmol/L lapatinib, and cell lysates were hybridized to a phospho-RTK array. In the array, each RTK is spotted in duplicate. Hybridization signals at the corners serve as controls. D. SNU216 and resistant (SNU216 LR4, LR6 and LR7) cell extracts were analyzed by western blot with antibodies recognizing phosphorylated and total MET, HER3, Stat3, Akt and MAPK. Data are representative of three independent experiments.

Figure 3.1A.

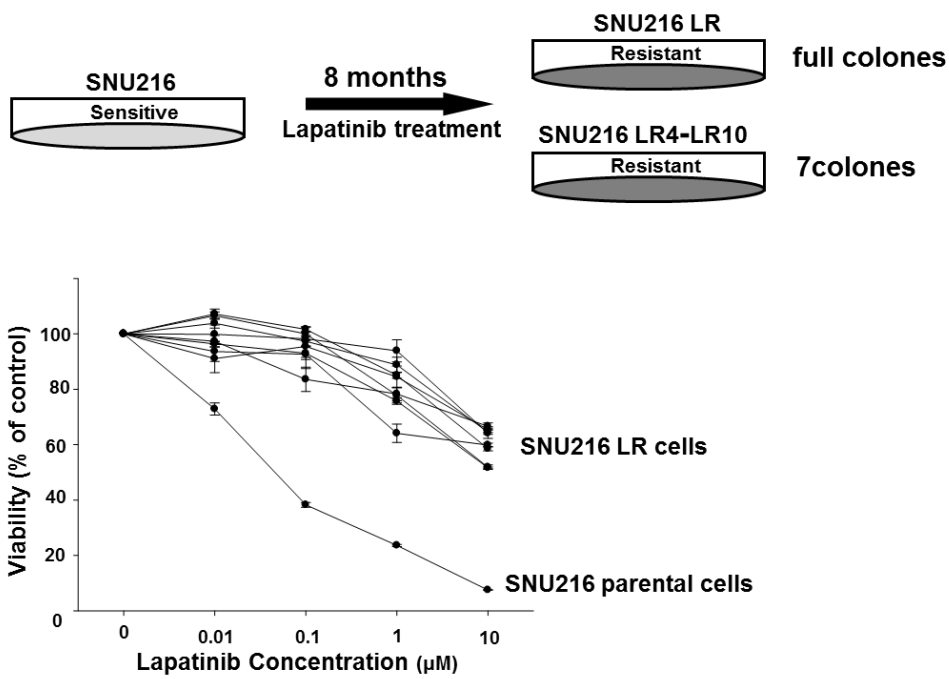

SNU216
Sensitive
8 months
Lapatinib treatment
SNU216 LR
Resistant
full colones
SNU216 LR4-LR10
Resistant
7colones
Viability (% of control)
100
80
60
40
20
0
0
0.01
0.1
1
10
Lapatinib Concentration (μM)
SNU216 LR cells
SNU216 parental cells

Figure 3.1B.

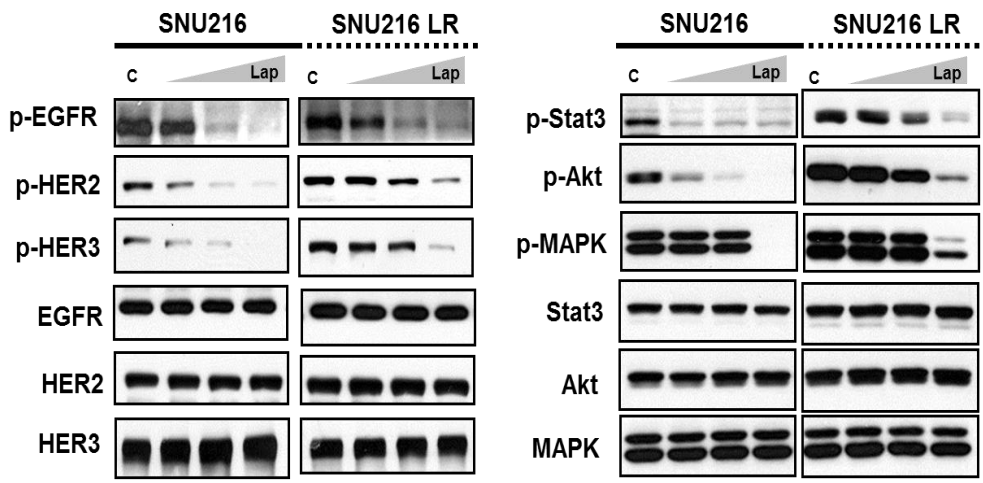

SNU216
SNU216 LR
C
Lap
p-EGFR
p-HER2
p-HER3
EGFR
HER2
HER3
SNU216
SNU216 LR
C
Lap
p-Stat3
p-Akt
p-MAPK
Stat3
Akt
MAPK

Figure 3.1C.

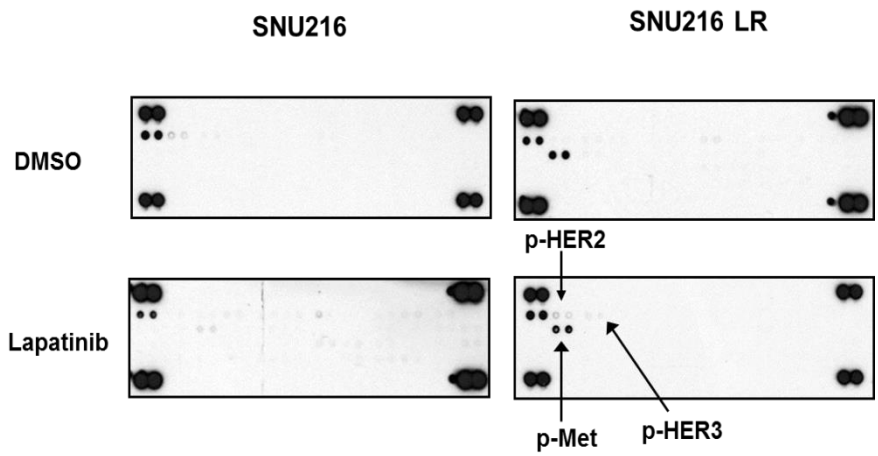


Figure 3.1D.

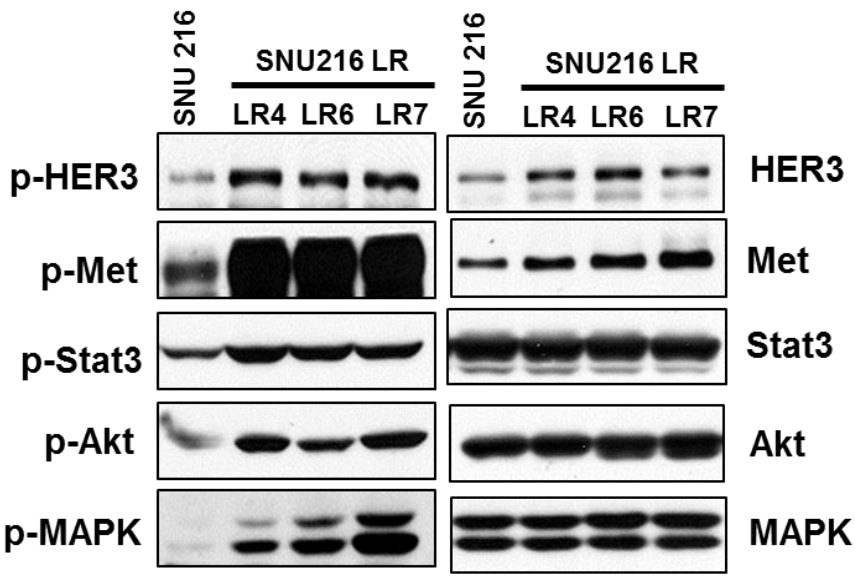

SNU 216
SNU216 LR
LR4 LR6 LR7
SNU 216
SNU216 LR
LR4 LR6 LR7
p-HER3
HER3
p-Met
Met
p-Stat3
Stat3
p-Akt
Akt
p-MAPK
MAPK

Figure 3.2. Lapatinib-resistant cell lines are resistant to growth inhibition by HER targeted inhibitors.

A. SNU216 and resistant cells lines (SNU216 LR4, LR6 and LR7) were treated with gefitinib (Gef), afatinib (Afa), dacomitinib (Daco), cetuximab (Cet) and trastuzumab (Trast) at indicated concentrations and the IC50 or IC30 values for HER targeted inhibitors was determined following 72 h of treatment. B. SNU216 and SNU216 LR4 cell lines were treated with gefitinib, cetuximab, lapatinib, afatinib and dacomitinib at 1 μmol/L for 24 h. Whole-cell extracts were analyzed by western blot with antibodies recognizing phosphorylated and total Stat3, Akt and MAPK. C. SNU216 and resistant cell lines (LR4, LR6 and LR7) were treated with 10 ng/ml trastuzumab for 24 h, and western blot was performed for both phosphorylated and total Akt and MAPK. Data are representative of three independent experiments.

Figure 3.2A.

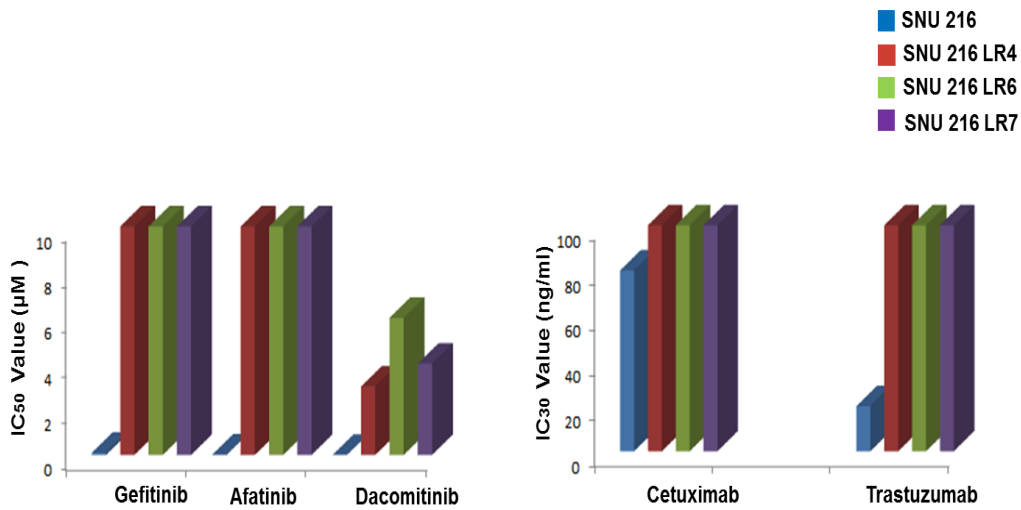

SNU 216
SNU 216 LR4
SNU 216 LR6
SNU 216 LR7
IC50 Value (μM)
0
2
4
6
8
10
Gefitinib
Afatinib
Dacomitinib
IC30 Value (ng/ml)
0
20
40
60
80
100
Cetuximab
Trastuzumab

Figure 3.2B.

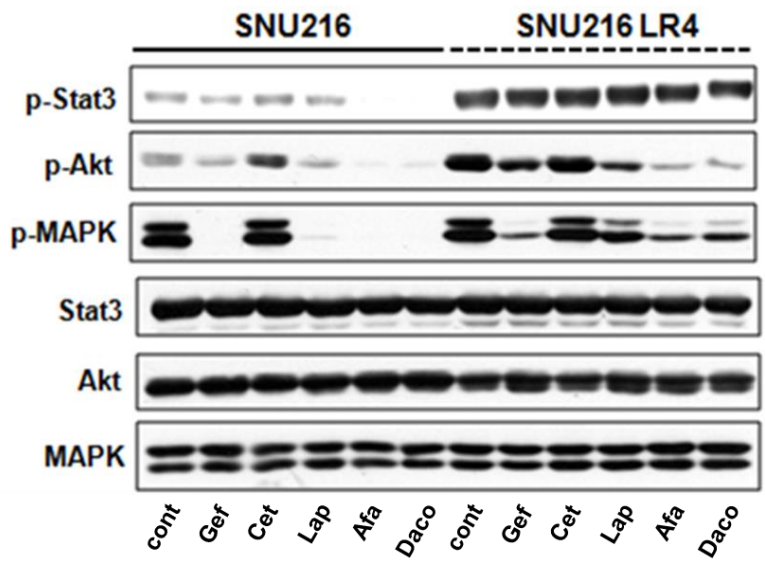

SNU216
SNU216 LR4
p-Stat3
p-Akt
p-MAPK
Stat3
Akt
MAPK
cont
Gef
Cet
Lap
Afa
Daco
cont
Gef
Cet
Lap
Afa
Daco

Figure 3.3. Lapatinib-resistant cell lines acquire an EMT phenotype. A. Gene expression signature from clustering analysis were depicted (red, significant enrichment of overexpressed genes; green, significant enrichment of underexpressed genes; yellow, not significant; $p<0.05$) B. Levels of Vimentin, Twist, Snail and Testican1 mRNA were determined via quantitative real-time RT-PCR. Differences in parental SNU216 versus resistant cells (LR4-LR10) are shown. Data are expressed as the mean ± SD, *$p < 0.05$. C. Western blot analysis for E-cadherin, vimentin, Testican-1 and -catenin are shown for SNU216 and resistant cell lines (LR4, LR6, LR7). D. Motility of SNU216 and resistant (LR4 and LR7) cells were measured by wound-healing assay. Data represent the mean ± SD (*$p<0.05$, SNU216 versus resistant cells) of three independent experiments**.**

Figure 3.3A.

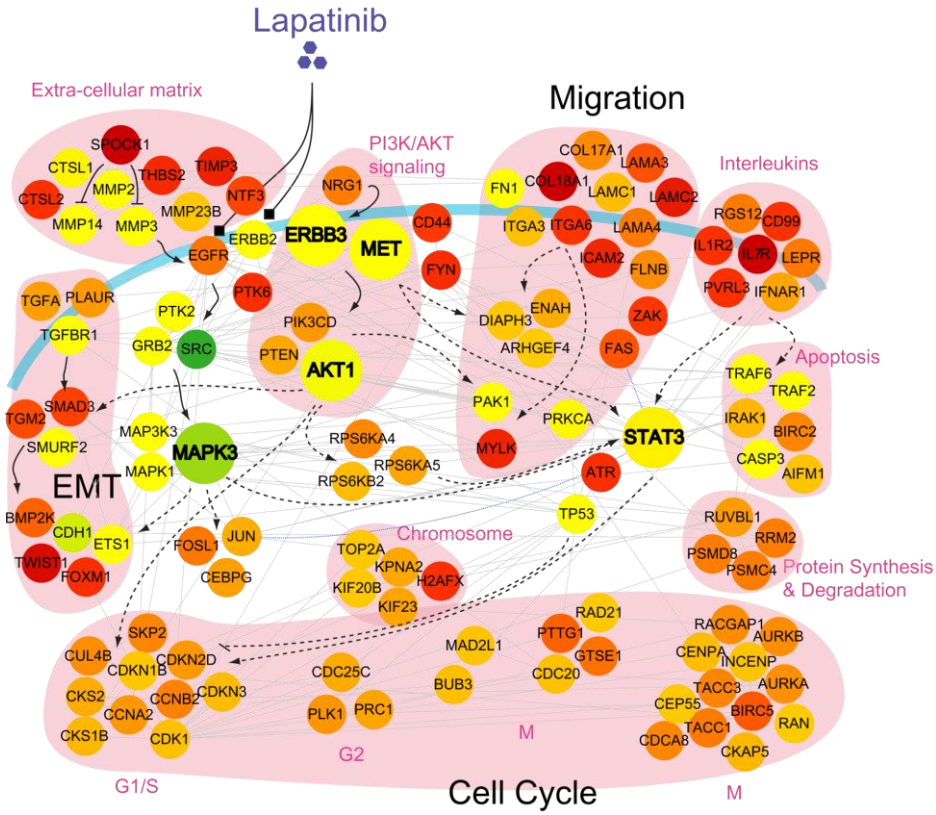

Lapatinib
Extra-cellular matrix
Migration
PI3K/AKT signaling
Interleukins
SPOCK1
CTSL1
CTSL2
MMP2
MMP14
MMP3
THBS2
TIMP3
NTF3
MMP23B
NRG1
ERBB2
ERBB3
MET
EGFR
CD44
FYN
COL17A1
LAMA3
FN1
COL18A1
LAMC1
LAMC2
ITGA3
ITGA6
LAMA4
ICAM2
FLNB
RGS12
CD99
IL1R2
IL7R
LEPR
PVRL3
IFNAR1
TGFA
PLAUR
TGFBR1
PTK2
PTK6
GRB2
SRC
PIK3CD
PTEN
AKT1
DIAPH3
ENAH
ARHGEF4
ZAK
FAS
Apoptosis
TRAF6
TRAF2
IRAK1
BIRC2
CASP3
AIFM1
SMAD3
TGM2
SMURF2
MAP3K3
MAPK3
MAPK1
EMT
RPS6KA4
RPS6KA5
RPS6KB2
PAK1
PRKCA
MYLK
STAT3
ATR
TP53
BMP2K
CDH1
ETS1
TWIST1
FOXM1
FOSL1
JUN
CEBPG
Chromosome
TOP2A
KPNA2
KIF20B
H2AFX
KIF23
RUVBL1
RRM2
PSMD8
PSMC4
Protein Synthesis & Degradation
SKP2
CUL4B
CDKN1B
CDKN2D
CKS2
CCNB2
CDKN3
CCNA2
CKS1B
CDK1
CDC25C
PLK1
PRC1
G2
MAD2L1
BUB3
RAD21
PTTG1
GTSE1
CDC20
M
RACGAP1
AURKB
CENPA
INCENP
TACC3
AURKA
CEP55
BIRC5
TACC1
RAN
CDCA8
CKAP5
M
G1/S
Cell Cycle

Figure 3.3B.

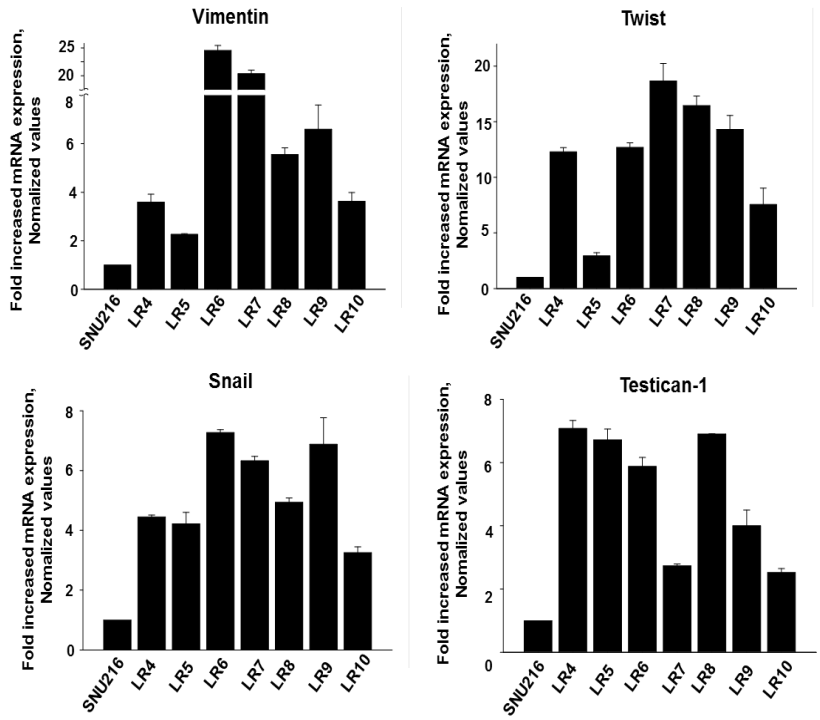

Vimentin
Twist
Snail
Testican-1
Fold increased mRNA expression, Nomalized values
SNU216
LR4
LR5
LR6
LR7
LR8
LR9
LR10

Figure 3.3C.

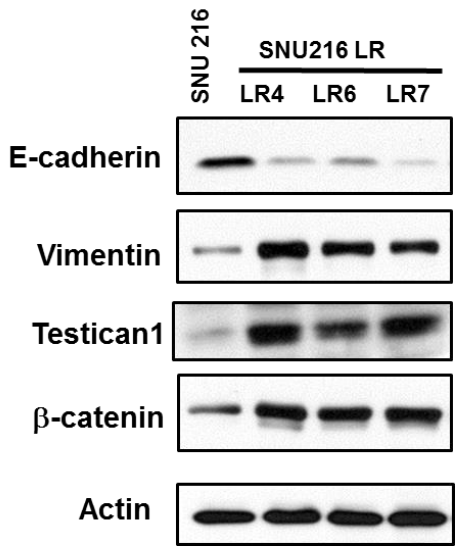

SNU 216
SNU216 LR
LR4
LR6
LR7
E-cadherin
Vimentin
Testican1
β-catenin
Actin

Figure 3.3D.

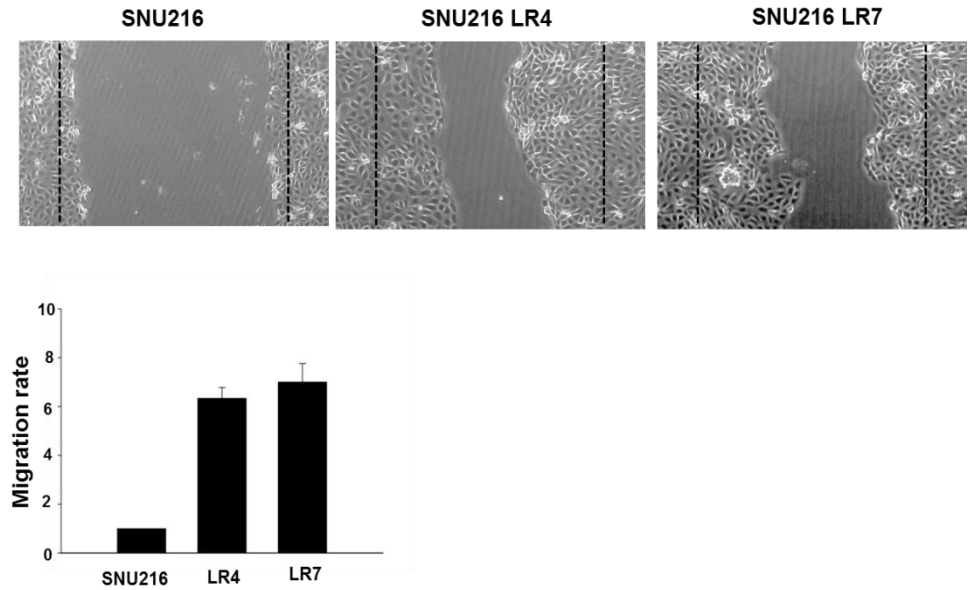

SNU216
SNU216 LR4
SNU216 LR7
Migration rate
10
8
6
4
2
0
SNU216
LR4
LR7

Figure 3.4. Silencing of Testican-1 induced MET dependent downstream signaling restores sensitivity to lapatinib.

A,B. SNU216 LR4 and LR7 cells were transfected with Testican-1 small interfering RNA (Si-Testican-1) or scramble siRNA (Si-cont) and viable cells were measured after 72 h. The expression of Testican1 was evaluated by real-time RT-PCR. Data represent the mean ± SD (*p<0.05,**p<0.05) of three independent experiments. Western blot analysis of phosphorylated and total Met, HER3, Vimentin, -catenin and -tubulin in SNU216 LR4 cells. C. SNU216 cells were transfected with plasmids expressing Testican-1 or control plasmid, and viable cells were measured after 48 h of lapatinib treatment (1 μmol/L). Data represent the mean ± SD (*p<0.01,**p<0.05) of three independent experiments.

Figure 3.4A.

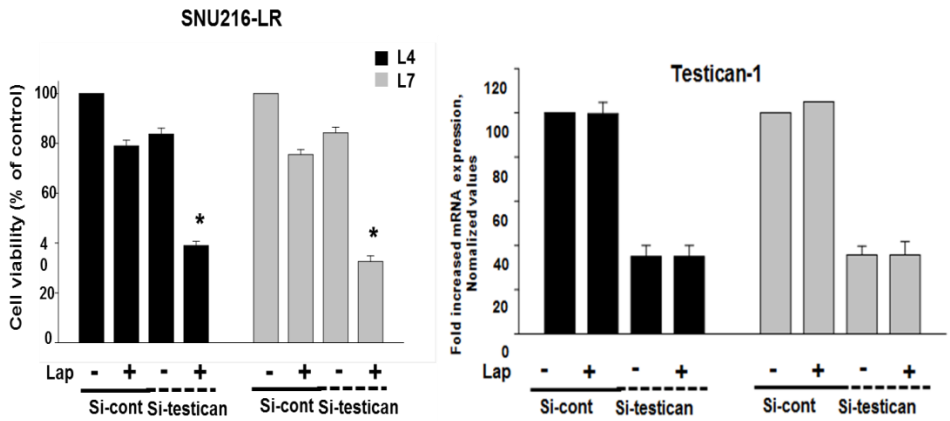

SNU216-LR
L4
L7
Cell viability (% of control)
100
80
60
4
0
20
0
*
*
Lap
- + - +
- + - +
Si-cont Si-testican
Si-cont Si-testican
Testican-1
Fold increased mRNA expression, Nomalized values
120
100
80
60
40
20
0
Lap
- + - +
- + - +
Si-cont
Si-testican
Si-cont
Si-testican

Figure 3.4B.

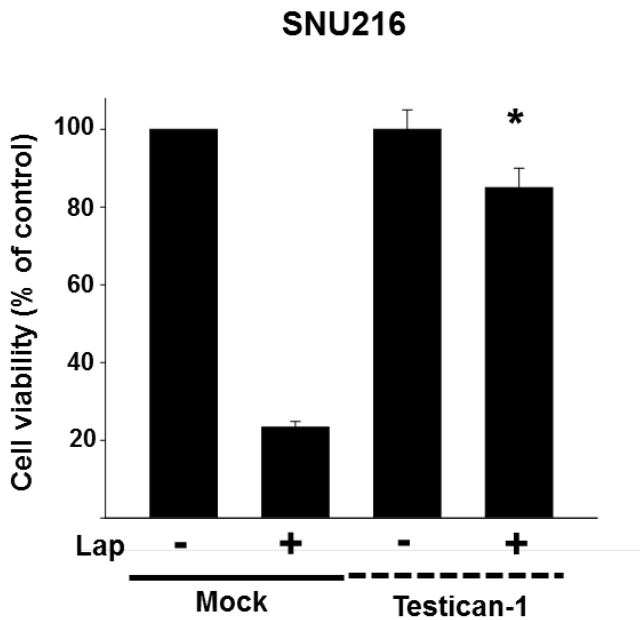

SNU216
Cell viability (% of control)
100
80
60
40
20
*
Lap
-
+
-
+
Mock
Testican-1

Figure 3.4C.

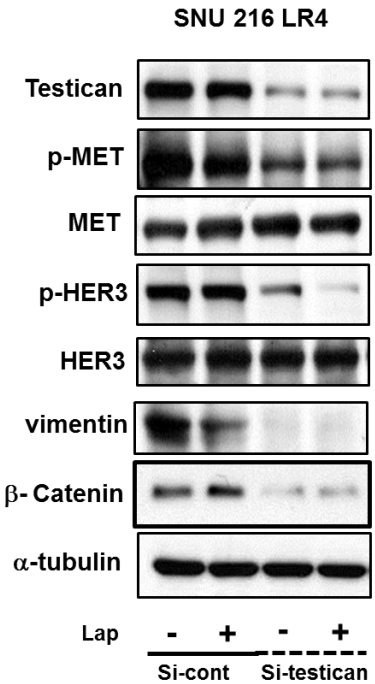

SNU 216 LR4
Testican
p-MET
MET
p-HER3
HER3
vimentin
β- Catenin
α-tubulin
Lap
- + - +
Si-cont
Si-testican

Figure 3.5. Combined treatment with XAV939 and lapatinib enhances cytotoxicity in lapatinib-resistant cells.

A. Dose-response analysis of XAV939 on the proliferation of SNU216 LR4 cells treated with lapatinib. Cells were treated with lapatinib (1 μmol/L) in combination with different doses of XAV939 (1, 5 or 10 μmol/L), and viable cells were measured after 72 h. Data represent the mean ± SD (*$p<0.01$, combination versus single) of three independent experiments. B. Combination index values were calculated using the CalcuSyn software. C. SNU216 LR4 cells were treated with lapatinib (1 μmol/L), XAV939 (1 μmol/L) or in combination at the indicated concentration for 48 h. Cells were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry. Proportions of cells in the G1, S and G2-M phase were quantified using the ModFit LT program (Verity Software House Inc.). D. Western blot analysis for β -catenin, cyclin D, p27, total and phosphorylated Stat3, Akt and MAPK. Data are representative of three independent experiments.

Figure 3.5A.

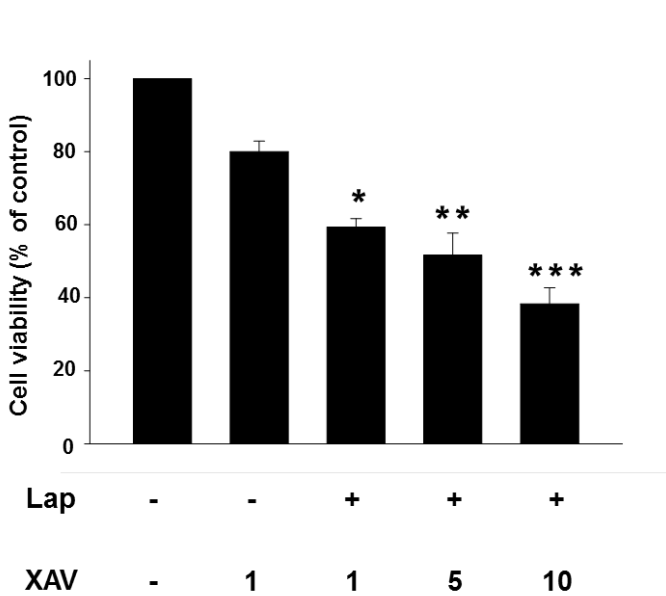


Figure 3.5B.

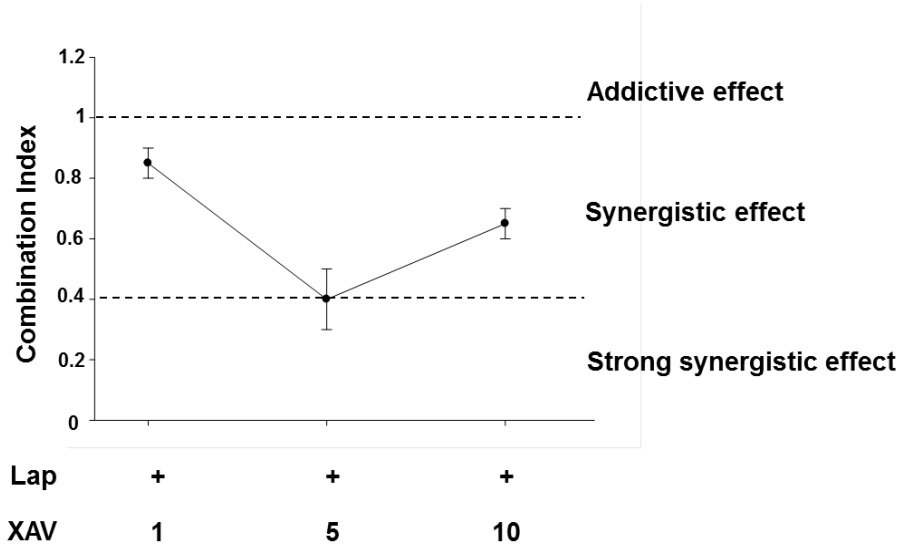

Combination Index
1.2
1
0.8
0.6
0.4
0.2
0
Addictive effect
Synergistic effect
Strong synergistic effect
Lap + + +
XAV 1 5 10

Figure 3.5C.

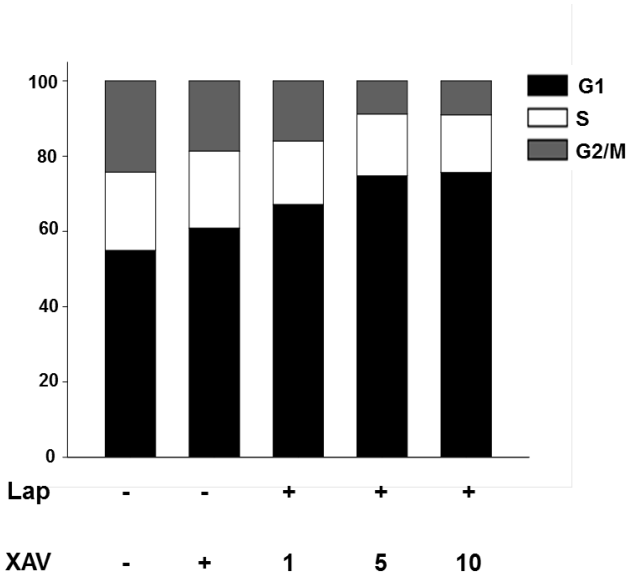
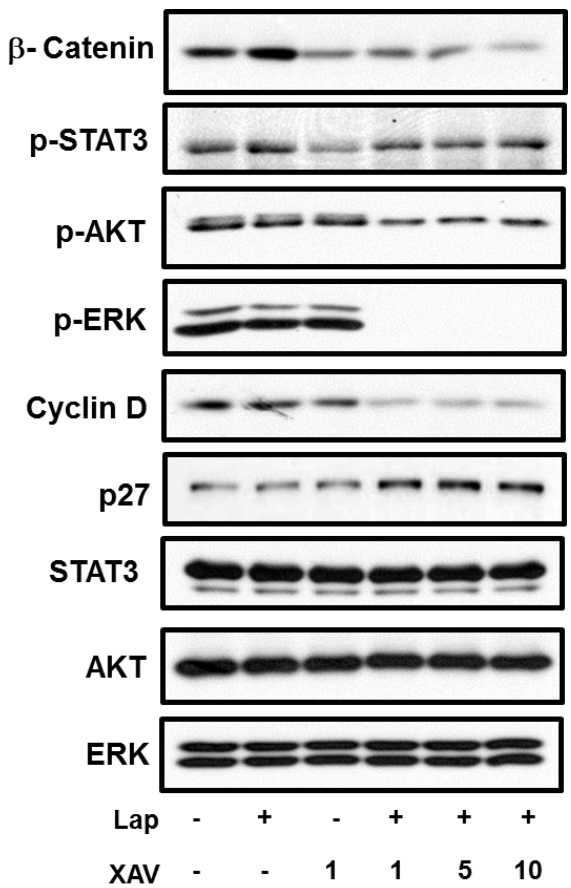


Figure 3.5D.


β- Catenin
p-STAT3
p-AKT
p-ERK
Cyclin D
p27
STAT3
AKT
ERK
Lap - + - + + +
XAV - - 1 1 5 10

Discussion

In this study, I identify a novel, molecular mechanism of acquired resistance to lapatinib, in HER2-positive gastric cancer cells. I established lapatinib-resistant cells from SNU216 HER2-positive gastric cancer cells in vitro, and characterized seven independent clones. In addition to displaying lapatinib resistance, these cell lines were also resistant to other HER-targeted agents, including trastuzumab (Figure 3.1B). Our data provide strong evidence that increased EMT signals confer acquired resistance to lapatinib in this model system. This mechanism of lapatinib resistance has not been previously identified in a HER2-positive gastric cancer model, although HER2 TKI-acquired resistant cell lines have been reported in a HER2-positive breast cancer model [87-89]. To date, there is evidence to indicate that resistance to HER2-targeted therapy in breast cancer cells is attributed to cross-talk with insulin-like growth factor receptor I [90], PTEN mutation or loss of heterozygosity [11, 91, 92], depression of FOXO3a with increased ER transcriptional activity [93, 94] increased membrane localization of HER3 [95] and extracellular domain shedding resulting in p95 [10, 96, 97]. The resistance has also been attributed to the contribution of other membrane

receptors or downstream effector(s). Recently, lapatinib resistance in BT474 human breast cancer cells, which are estrogen receptor-positive, progesterone receptor-positive and HER2-positive, was shown to be the result of AXL activation. It was suggested that this may represent an alternative pathway for proliferation, capable of bypassing the HER2 signaling pathway. However, such molecules associated with resistance to HER2 therapy were not identified in our gastric cancer model. This study is the first to suggest the acquired-resistance mechanism of lapatinib in HER2-positive gastric cancer.

Recent studies have shown that resistance to EGFR-TKI in NSCLC cell lines is associated with EMT. EMT is linked to the loss of cell-cell adhesion, cellular elongation and invasion of the underlying ECM. EMT progression is characterized by the loss of proteins involved in cell junctions, such as E-cadherin and the claudins, and the expression of mesenchymal markers, such as vimentin. I previously reported that TM4SF5-mediated EMT and regulation of activity or integrity of membrane receptors including EGFR, IGF1R and/or MET on the cell surface, is likely important for gefitinib resistance in cancer cells. In this study, I generated novel, lapatinib-resistant, variant sub-lines from SNU216 parental cells by repetitive, dose-escalating lapatinib treatment in

vitro. Interestingly, these lapatinib-resistant lines (SNU216 LR) consist of only fibroblast-like tumor cells and show typical characteristics of EMT, such as the marked decrease in E-cadherin, increased Vimentin and Snail expression and increased cell motility. Moreover, using microarray analysis, I demonstrate that lapatinib-resistant cells display upregulation of EMT-associated gene signatures. I also show that Testican-1-induced MET-dependent activation of EMT signaling occur resistant to lapatinib in HER2-positive gastric model. Thus, I present the first EMT line of HER2-positive gastric cancer, displaying resistance to HER2-targeting agents, providing a useful in vitro model to understand the mechanism underlying the link between EMT and lapatinib resistance.

Many signaling pathways, including Wnt/β-catenin, Notch, TGF-β and signaling pathways, have been shown to be critical for EMT induction. The activation of Wnt/β-catenin signaling is known to regulate the expression of specific target genes and plays important roles in growth and development, including the regulation of proliferation and apoptosis. Therefore, not surprisingly, alterations in β-catenin signaling are associated with tumorigenesis. Moreover, it has previously been reported that β-catenin is involved in EMT induction during tumor progression, converting polarized,

epithelial cells into motile, invasive cells. In hepatocytes, β-catenin can be released from an additional membrane-bound pool associated with the MET receptor. When engaged by HGF, MET releases β -catenin into the cytoplasm, leading to its eventual translocation into the nucleus. Our results are consistent with these findings. On the basis of our findings, I propose that inhibition of Testican-1 induced EMT signaling may enhance response to lapatinib treatment in appropriately selected HER2-positive gastric cancer patients.

In this study, the measurement of basal gene expression levels allowed us to identify Testican-1 mediated EMT as a mechanism of acquired resistance to lapatinib in HER2-gastric cancer cells. Testican-1 silencing partially restored sensitivity to lapatinib in resistant cells, implying that other signaling pathways may also be responsible for drug resistance. However, owing to the limited number of cell lines used in our experiments, it is possible that other important biological factors exist that contribute to drug resistance in HER2-positive gastric cancer.

In summary, our work demonstrates that HER2-positive gastric cancer cells that develop resistance to lapatinib may also develop cross-resistance to several other HER2-targeted agents. Acquired resistance to lapatinib was

associated with EMT. While these molecular changes may serve as molecular markers of resistance, they may also be associated with an increased invasive capacity of cancer cells in vitro. Our study may assist in understanding the mechanisms associated with acquired resistance to lapatinib, and serve to identify resistant tumors in clinical trials. SNU216-LR cells also represent a potentially useful tool for gaining insights into the mode of action of lapatinib in tumors, in addition to investigating and developing methods to prevent resistance to this drug or other targeted therapies.

Ⅴ. Conclusion

1. Lapatinib downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2

I have demonstrated that lapatinib, a dual inhibitor of EGFR and HER2 TK, effectively downregulates a variety of nucleotide synthesis-related genes, including TS, and exhibits activity superior to that of gefitinib not only in HER2-amplified cells, but also in wild-type cells. As a mechanism, I have determined, for the first time, that nuclear EGFR and HER2 activate TS gene transcription, and that EGFR and HER2-bound TS promoter activities are inhibited by lapatinib treatment

2. Combined lapatinib and cetuximab enhance cytotoxicity against gefitinib-resistant lung cancer cells

I propose that combination of lapatinib and cetuximab is mechanistically cooperative because lapatinib affects receptor heterodimerization and cetuximab primarily reduces the level of EGFR proteins. In gefitinib-resistant T790M lung cancer cells, lapatinib reduced Stat3 activation by blocking the heterodimerization of EGFR and HER2, and cetuximab caused a complete down-regulation of EGFR. The combination of lapatinib and cetuximab

completely blocked the activation of downstream signals including Stat3, resulting in enhanced cytotoxicity against in T790M cells. Therefore, this combination provides a therapeutic concept of a complete blockade of EGFR signaling for cancer therapy. Because both drugs are currently being used in clinics, these findings can be directly tested in future clinical investigations.

3. Testican-1 mediated epithelial-mesenchymal transition signaling confers acquired resistance to lapatinib in HER2-positive gastric cancer

HER2-positive gastric cancer cells that develop resistance to lapatinib may also develop cross-resistance to several other HER2-targeted agents. Acquired resistance to lapatinib was associated with EMT. While these molecular changes may serve as molecular markers of resistance, they may also be associated with an increased invasive capacity of cancer cells in vitro. Our study may assist in understanding the mechanisms associated with acquired resistance to lapatinib, and serve to identify resistant tumors in clinical trials**.**

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국문 초록

상피 성장 인자 수용체(epidermal growth factor receptor, EGFR)은 대부분의 상피성 종양에서 과발현 되는 tyrosine kinase 수용체이다. 이 수용체는 암 치료의 대상으로 제안된 최초의 수용체이며, 집중적인 연구의 결과로 임상에서 사용할 수 있는 각종 EGFR 저해제들이 개발되어 다양한 암종에서 사용되고 있다. 특히, EGFR 타깃 항암제들의 감수성 또는 저항성 예측인자, 즉 바이오마커 개발과 관련 기전을 규명하는 연구는 일부 특정암에서 연구가 되었고, 현재에도 여러 연구실에서 기전규명 연구가 계속 진행되고 있다. 본 연구자는 위암, 폐암대상으로 표적 항암제인 lapatinib과 gefitinib의 감수성과 내성 바이오마커 개발과 그 기전 규명연구를 수행하였다.

제 1연구에서는 HER2 증폭된 위암에서 lapatinib 항암제는 nuclear EGFR과 HER2의 translocation을 저해함으로 thymidylate synthetase (TS)를 조절하는 기전을 규명한 연구로, EGFR 저해제와 fluoropyrimidine 항암제의 병용은 특정 암에서 상승효과가 있음이 알려졌으나, 그 기전 연구는 전무하다. 본 연구자는 위암에서 lapatinib 항암제와 fluoropyrimidine 항암제의 병용시 상승효과의 기전을 연구 하였다.

cDNA micro array 결과에서 lapatinib에 의해 TS 유전자를 포함한, nucleotide synthesis 연관된 유전자들의 발현 감소되는 것을 확인하였다. 흥미 있는 것은 lapatinib 항암제 처리시, EGFR과 HER2 유전자가 핵속으로 translocation 되는 것이 저해되는 것을 발견하였고, 특히 nuclear EGFR과 HER2의 존재와 그 역할은 transcription factor로 작용함으로 TS유전자를 조절함을 관찰하였다. 이는 위암에서 최초로 lapatinib 항암제와 fluoropyrimidine 항암제의 병용효과와 그 기전을 규명하였고, 이런 결과는 HER2 증폭된 위암 환자중 항암제 내성이 있는 환자에게 맞춤형 항암요법으로 쓰일 수 있는 치료 전략을 제시한 연구 결과이다.

제 2연구에서는 gefitinib 항암제에 대한 저항성이 있는 폐암에서 lapatinib 항암제와 cetuximab 항암제의 병용효과와 그 기전을 규명한 연구이다. Gefitinib 항암제의 치료를 받은 환자중 내성이 있는 환자에게서 EGFR 유전자의 tyrosine kinase domain 에 T790M 돌연변이가 있음을 발견하였다. 본 연구자는 EGFR T790M 이 발현하는 세포주를 수립하였고, 이 모델을 이용하여 T790M 획득 돌연변이에 의하여 gefitinib 항암제에 대한 내성이 발생됨을 관찰하였다. 특히, EGFR T790M 돌연변이에 의해 stat3 유전자가 활성화 됨을 발견하였고, 이는 lapatinib 항암제 처리시, EGFR과 HER2 유전자의 dimerization을 저해함으로서 하부신호 유전자인 stat3 활성화를 저해하여 암세포 성장을 억제함을 관찰하였다. 또한,

cextuximab 항암제와 lapatinib 항암제의 병용시, 상승효과가 있음을 *in vitro/in vivo* 실험으로 확인하였고 그 기전은 cetuximab에 의한 EGFR degradation과 lapatinib에 의한 EGFR과 HER2 dimer-dissociation을 통한 stat3 활성화를 저해함으로 상승효과가 나타남을 보여 주었다. 이런 결과는 gefitinib 항암제에 대한 내성을 예측하는 바이오마커로 임상에서 쓰일 수 있고, 폐암 환자의 맞춤형 항암요법에 대한 치료 전략을 보여주는 연구결과이다.

제 3연구는 HER2 증폭된 위암에서 Testican-1 유전자에 의한 EMT (Epithelial-mesenchymal transition) 현상이 lapatinb 항암제에 대한 내성을 발생시킴을 보여 주는 연구이다. 위암에서 lapatinib 항암제의 획득 내성 관련 기전연구는 전무하여, 본 연구자는 8개월 동안 HER2 증폭된 위암세포주에 lapatinib항암제를 지속적으로 처리하여 최초로 lapatinib에 대한 내성 위암 세포주를 수립하였다. 내성 세포주에서는 HER3 유전자와 MET 유전자가 과발현 되어 있는 것을 관찰하였고, 특히 이 세포는 다른 EGFR 타깃 항암제에게도 내성을 나타냄을 확인 하였다. 이 모델을 사용한 cDNA microarray 결과, 내성 세포주에서 Testican1 유전자를 포함한 EMT 관련 유전자들의 발현 증가와 EMT 현상 증가가 관찰되었다. 흥미로운것은 Testican-1 발현 억제후, HER3과 MET 유전자의 활성 감소와 EMT 현상 감소는 lapatinib 항암제에 대한 내성을 회복시키는 것을 확인

하였다. 또한, EMT 억제재를 처리하였을 경우, 동일 현상을 나타내며 lapatinib 내성세포의 세포성장을 억제하는 것을 관찰하였다. 이것은 HER2 증폭 위암에서 최초로 lapatinib 항암제의 내성을 발생시키는 기전을 규명한 연구로, Testican-1 유전자에 의해 야기되는 EMT 현상은 lapatinib 내성을 유도함을 보여주는 연구이다. 이런 결과는 HER2 증폭 위암 환자의 맞춤형 항암요법에 중요한 치료 전략으로 사용될 수 있다.

본 연구자는 폐암, 위암 대상으로 한 본 연구는 EGFR 타깃 표적항암제인 lapatinib과 gefitinib의 감수성/저항성 예측 바이오마커로 실제 임상에서 중요한 자료로 쓰일 수 있고, 그 기전 연구는 EGFR 타깃 치료연구의 단서를 제공해 줄 수 있다고 생각한다.

주요어 : EGFR, HER2, gefitinib, lapatinib, cetuximab, gefitinib 내성, Testican-1, epithelial-mesenchymal transition, lapatinib 내성.

학번: 2002-23756

감사의 글

Cancer biology를 처음 접하면서부터 지금까지 아낌없이 지도하여 주시고, 세심하게 논문의 방향을 제시하여 주신 방영주 교수님께 머리 숙여 깊은 감사를 드립니다. 그리고 논문의 세심한 부분까지 잘펴 주시고, 진정한 과학자로서의 자세를 깨닫게 해 주신 김태유 교수님, 부족한 부분을 자상히 지적해 주시고 항상 후학의 앞을 걱정해 주시는 임석아 교수님, 논문이 완성 될 수 있도록 도움을 주시고 따듯한 말을 아끼지 않으셨던 오도연 교수님, 그리고 항상 옆에서 연구에 대한 조언을 해주신 영원한 친구 한세원 교수님께 감사를 드립니다. 또한, 부족한 논문을 세심하게 심사해 주신 전용성 교수님, 송용상 교수님과 고려대학교 김열홍 교수님게 감사의 말씀을 드립니다.

생물학의 기초부터 연구 방법까지 모든 부분을 아낌없이 가르쳐 주신 이정원 교수님께도 감사를 드립니다. 언제나 선배님으로서 격려의 말씀과 조언을 아끼지 않으신 김상균박사님, 힘들때 마다 걱정하여 주시고 위로해 주신 송상현박사님, 박사는 모든 분야에 박학다식하여야 함을 보여 주신 박정현 박사님, 논문을 너무 많이 읽어 박사가 된 친구 성학이, 나보다 어른스러운 용배, 친 동생 같은 후배 미숙이, 성률이에게 감사의 말을 전합

니다. 얼마전 결혼해서 나한테 소홀해진 영광이, 그리고 많은 실험실 후배들에게 마음으로부터 깊은 감사를 전합니다.

무엇보다도 저를 항상 자랑으로 여기시고, 처음부터 끝까지 조건 없는 사랑을 주시는 부모님, 물심양면 도와 주신 누나와 매형께 감사 드립니다.

지금이 있기까지 아무말 없이 내조해 준 아내, 나현과 너무 이쁜 딸, 효린에게 사랑을 전합니다.

끝으로 그 동안의 연구결과가 밑거름이 되어 앞으로의 훌륭한 연구 성과와 암 정복이 되는 날이 하루 빨리 오기를 기원 합니다.