

Original Article

Transglutaminase 2 induces intrinsic EGFR-TKI resistance in NSCLC harboring EGFR sensitive mutations

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Abstract: The non-small cell lung cancer (NSCLC) patients with EGFR-sensitive mutations can be therapeutically treated by EGFR-TKI such as erlotinib and gefitinib. However, about 40% of individuals harboring EGFR-TKI sensitive mutations are still resistant to EGFR-TKI. And, it has been reported that both PTEN loss and NF- κ B activation contribute to intrinsic EGFR-TKI resistance in EGFR-mutant lung cancer. Transglutaminase 2 (TG2) is post-translational modification enzyme and known to induce degradation of tumor suppressors including PTEN and I κ B α with peptide cross-linking activity. Because TG2 was known as a regulator of PTEN and I κ B α (NF- κ B inhibitor) level in cytosol, we have explored if TG2 can be another key regulator to the intrinsic resistance of EGFR-TKI in the intrinsic EGFR-TKI resistant NSCLC cell. We first found that higher TG2 expression level and lower PTEN and I κ B α expression levels in the intrinsic EGFR-TKI resistant NSCLC compare with EGFR-TKI sensitive NSCLC. TG2 stably expressing EGFR-TKI sensitive NSCLC cells harboring EGFR mutations showed reduction of both PTEN and I κ B α and exhibited EGFR-TKI resistance. In reverse, When TG2 is downregulated by TG2 inhibitor in H1650, intrinsic EGFR-TKI resistant NSCLC cell harboring EGFR sensitive mutation, reversed EGFR-TKI resistance via I κ B α restoration. Moreover, combination treatment of TG2 inhibitor and EGFR-TKI decreased the tumor growth in mouse xenograft models of EGFR mutant NSCLCs. Therefore, we have demonstrated that TG2 elicits the intrinsic EGFR-TKI resistance via PTEN loss and activation of NF- κ B pathway. These results suggest that TG2 may be a useful predictive marker and also be a target for overcoming the resistance.

Keywords: Non-small cell lung cancer, transglutaminase 2, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), combination therapy, drug resistance

Introduction

Epidermal growth factor receptor (EGFR) is a 486 amino acid, 170-kDa receptor protein with a single transmembrane sequence. It was first identified as a receptor of EGF. EGFR displays tyrosine kinase (TK) activity. In the early 2000's,

the first two EGFR TK inhibitors (TKIs), gefitinib and erlotinib, were initially developed to inhibit the EGFR signaling pathway by blocking the intracellular TK domain [1-3]. During the clinical development of EGFR-TKIs, somatic mutations in this TK domain were discovered in patients with advanced or metastatic non-small cell lung

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cancer (NSCLC) [4], and were reported to correlate with high response rates of EGFR-TKI therapy. Clinical trials of EGFR-TKI therapy showed the superiority in survival outcome to standard cytotoxic chemotherapy. Therefore, currently, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) is a standard treatment for non-small cell lung cancer (NSCLC) patients with EGFR sensitizing mutations based on its high response rates, longer progression-free and overall survival as well as good safety profile.

This identification of “driver mutation” and “actionable alteration” was a breakthrough in molecular-targeted therapy and helped change the paradigm of cancer treatment [5, 6]. However, the initial hope of EGFR-TKI therapy was soon blunted by the phenomenon of “acquired resistance” to the inhibitor, which developed inevitably after a median response duration of 9 to 13 months [7]. Recently, 3rd-generation EGFR TKI, such as osimertinib, showed better survival outcomes than prior 1st or 2nd-generation EGFR TKIs but it also inevitably showed primary or acquired resistance. In addition, EGFR-TKI therapy induced response in approximately 70% of patients but the other 30% did not respond to EGFR-TKI therapy. This was termed “primary resistance”, and occurred even with the presence of an activating or actionable mutation [8]. An area of active research is the development and mechanism of acquired resistance, since the knowledge will help choose treatment options after EGFR-TKI treatment. However, the mechanisms of intrinsic EGFR-TKI resistance in patients harboring EGFR sensitive mutations are relatively unknown. Several primary resistance mechanisms are known and include KRAS mutation, T790M mutation, and Bcl-2-like protein 11 (BIM) polymorphism [9]. Loss of phosphatase and tensin homolog (PTEN) contributes to intrinsic EGFR-TKI resistance in EGFR mutant lung cancer through activation of the phosphoinositol-3-kinase/protein kinase B (PI3K/AKT) pathway [10-12]. Recently, Cripto-1 was known to induce intrinsic EGFR-TKI resistance in NSCLC as well [13]. Activation of nuclear factor-kappa B (NF- κ B) is also involved in EGFR-TKI intrinsic resistance in EGFR mutant lung cancer [14, 15]. In 2015, Blakely et al. revealed that NF- κ B signaling is extremely activated early in response to EGFR inhibition in lung cancer. They

also showed continuous NF- κ B activation after EGFR-TKI treatment drives tumor cell survival and resistance. In addition, NF- κ B inhibitor restore this EGFR-TKI sensitivity and enhance patient outcomes in EGFR-TKI resistant NSCLC [15].

Transglutaminase 2 (TG2) is a multi-functional enzyme that is ubiquitously expressed as a cytosolic protein, but also at the cell surface and in the extracellular matrix [16] in mammalian tissues. TG2 is related to diverse cellular processes. TG2 catalyzes calcium ion-dependent post-translational protein modification by unalterable ϵ (γ -glutamyl) lysine cross-links between polypeptide chains. This crosslinking activity of TG2 forms oligomers of substrate proteins, which are finally removed by proteasome-mediated protein degradation [17]. Therefore, TG2 has been associated with various physiological and pathological conditions, such as signal transduction, apoptosis, cell adhesion, cell migration, extracellular matrix formation [18-20], wound healing [21], tumor progression and metastasis [22-24], fibrosis [25, 26], and bone formation [27].

In addition, during the past two decades, a number of studies has revealed that TG2 is highly expressed in pancreatic cancer [28], breast cancer [29, 30], colon cancer [31], ovarian cancer [32], non-small cell lung cancer (NSCLC) [33], glioblastoma [34], and melanoma [35]. Increased TG2 expression is frequently involved with drug resistance and metastasis [29, 36-39]. Particularly, TG2 inhibition by inhibitors, including small interfering RNA (siRNA) and short hairpin RNA (shRNA) can restore drug sensitivity in resistant tumors and sensitize drug-resistant cancer cells [38, 40]. TG2 exerts its anti-apoptotic effects in various cancers via different mechanisms including transamidation activity and GTP binding activity [40-42]. As a representative example, the crosslinking activity of TG2 induces polymerization of several tumor suppressors, including PTEN and I κ B α , with removal by ubiquitin-dependent proteasomal degradation. The crosslinking function of TG2 protects tumor cells from caspase cleavage and supports cancer cell survival by NF- κ B activation [40, 41, 43, 44]. The collective findings indicate that TG2 could be a potent anticancer target and some TG2 inhibitors have been developed and are

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being prepared for pre-clinical trials by pharmaceutical companies [45, 46].

As mentioned above, TG2 induces PTEN loss and activates NF- κ B via I κ B α depletion, which comprises intrinsic EGFR-TKI resistance. Presently, we investigated whether TG2 plays a role in resistance against EGFR TKI therapy, what mechanism involves in the resistance and how the resistance could be overcome, hoping to develop new strategies maximizing the efficacy of the current EGFR TKI therapy.

Material and methods

Cell culture and reagents

The human NSCLC cell lines, H1650, HCC827, and H4006, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured by incubation in an atmosphere of 5% CO₂ at 37°C in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Waltham, MA, USA). Glucosamine was purchased from Sigma-Aldrich (St Louis, MO, USA) and diluted with autoclaved water to 200 mM. Erlotinib (CP358774), osimertinib (AZD9291) and omipalisib (GSK458) were purchased from Selleck Chemicals (Houston, TX, USA). Each was dissolved in dimethyl sulfoxide to 10 mM.

Cell viability assay

Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) following the manual instructions. Approximately 3×10^3 cells were plated to white 96 well plates. The next day, the culture medium was removed and the desired concentrations of glucosamine or/and erlotinib (CP358774) or osimertinib (AZD9291) were treated to a volume of 100 μ l. After 72 hours, 100 μ l of CellTiter-Glo reagent was added and the plates were incubated for 10 minutes at room temperature. The luminescence was measured using a Wallac 1420 apparatus (Perkin-Elmer, Boston, MA, USA).

Transfection

To overexpress TG2, the pcDNA3.1/TG2 vector was transfected in HCC827 and H4006 cells. Transfection was implemented using Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad,

CA, USA) following the manufacturer's protocol. HCC827/TG2 and H4006/TG2 cells were established by G418 selection (500 mg/ml; Gibco, Carlsbad, CA, USA). To knockdown TG2 in H1650 cell line, transfection of TG2 short interfering RNA (TG2 siRNA, Sense; 5'-CCAAGUUCAUCAAGAACAUUU-3', Anti-sense: 5'-AUGUUCUUGAUGAACUU GGUU-3') were performed by using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Western blot analysis

All cultured cells were washed twice with ice-cold PBS and harvested. The harvested cells were lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor (1 mM sodium fluoride and 2 mM sodium orthovanadate). The lysed samples were stored on ice for 30 minutes and centrifuged at 13000 g for 30 minutes. The supernatant was collected and the total protein of the whole cell lysate was determined. A volume containing 10-20 μ g of protein was subjected to 8-15% SDS-PAGE and the resolved proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). After blocking, the membrane was exposed to primary antibody overnight at 4°C and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) in TBS-T containing 1% bovine serum albumin for 1 hour at room temperature. The proteins were visualized using ECL Plus enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) and G-box Chemi Systems (SynGene, Bangalore, India). TG2 antibody was obtained from ThermoFisher Scientific (CUB 7402, Waltham, MA, USA). The other antibodies, including AKT, phosphorylated (p)-AKT, ERK, p-ERK, BIM, PARP, I κ B α , and β -Actin, were purchased from Cell Signaling Technology (Danvers, MA, USA).

Xenograft study

In order to confirm our theory, we used mouse xenograft model because this in vivo experiment is normally used for anti-cancer effect before human treatment [47]. Six-week-old female BALB/c-nu/nu mice were purchased from Central Lab and Animal Inc. (Seoul, Korea) and were used as a model for in vivo experiments [47]. Twelve light/twelve dark cycle and

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23°C with 40-60% humidity are used for mice housing. After one week of adaptation with woodchip bedding and cage enrichments (Nestlets) in specific pathogen free (SPF) facility, 1×10^7 HCC827, HCC827/TG2, and H1650 cells were subcutaneously implanted in the isoflurane-anesthetized mice right flank, respectively [48]. Approximately 2 weeks later, when the average tumor volume reached 100 mm³, the mice were randomly divided into groups of three mice per cage. Glucosamine (500 mg/kg) was administered intraperitoneally and erlotinib (25 mg/kg) was given orally every day for 21 days. In order to minimize the effects of subjective bias, principal investigator labeled drug names as A, B, C and D to make tester unaware. Tumor volumes were monitored for 3 weeks (measured every 3 days) by caliper and volume (mm³) was calculated using the formula $V = (W^2 \times L)/2$ for caliper measurements. W is tumor width, L is tumor length, respectively. After 3 weeks, all animals were sacrificed by carbon dioxide (AVMA Guidelines for the Euthanasia of Animals). All mice in each group included for each analysis. All Animal procedures were approved by the Asan Medical Centre Institutional Animal Care and Use Committee and Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

Caspase 3/7 assay

Caspase 3 and 7 activation was measured by using the Caspase-Glo 3/7 Luminescence Assay (Promega Corp. Madison, WI, USA) following the manufacturer's protocol. Protein samples from cells were prepared using RIPA buffer in the same manner as western blot sample preparation. Tumor samples of the xenograft models were homogenized and proteins were extracted as previously described [49]. Ten micrograms of protein samples in 100 µl total volume were transferred to white 96 wells. One hundred microliters of equilibrated Caspase-Glo 3/7 Reagent were added to protein samples and incubated for 1 hour at room temperature. Luminescence was measured by using the Wallac 1420 apparatus (PerkinElmer, Waltham, MA, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples from patients harboring EGFR mutants were obtained from Asan Medical Center (AMC, Seoul, Korea). Immunohistochemistry was performed

using Ultra-Sensitive ABC Peroxidase Staining kits (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Deparaffinized samples were stained with TG2 primary antibody (ThermoFisher Scientific, CUB 7402, Waltham, MA, USA) diluted to 1 µg concentration at 4°C overnight. After primary antibody staining, biotinylated secondary antibody and ABC Reagent were sequentially added to samples and reacted at room temperature for 30 minutes. Samples were reacted with substrate using AEC Substrate Chromogen (K3464; Dako, Carpinteria, CA, USA). Finally, counterstaining was done using hematoxylin (S3309, Dako, Carpinteria, CA, USA).

Results

TG2 overexpression is related with primary EGFR-TKI resistance in EGFR-mutated NSCLC cells

Previous reports already established that HCC827, H4006 and H1650 cell lines are known to harbor EGFR sensitizing mutations. These three cell lines are supposed to sensitive to EGFR-TKI because they have EGFR sensitive mutants [13]. However, H1650 cells are resistant to EGFR-TKI, even though the cells harbor an EGFR sensitive mutation, exon 19 deletion [50]. Actually, H1650 has deleted PTEN gene as well as mutated EGFR gene. We confirmed that H1650 cells display intrinsic or primary resistance to erlotinib, a first-generation EGFR-TKI, among EGFR mutant NSCLC cell lines via CellTiter-Glo luminescent cell viability assay compared with HCC827 and H4006 cells harboring EGFR sensitive mutants (**Figure 1A**). TG2 is highly overexpressed in H1650 cells but not in other sensitive cell lines (**Figure 1B**). Interestingly, H1650 cells displayed loss of PTEN and IκBα, well-known substrates of TG2, and activation of AKT, which is a well-known downstream pathway of TG2 (**Figure 1B**), compared with HCC827 or H4006 cells harboring EGFR mutants (**Figure 1A**). In addition, we observed that H1650 cells remained resistant to osimertinib, a third-generation EGFR-TKI (**Figure 1H and 1G**).

TG2 renders EGFR-mutated NSCLC cells resistant to EGFR-TKIs via activation of both AKT and NF-κB

We then induced ectopic TG2 expression in EGFR-TKI sensitive HCC827 cells in order to

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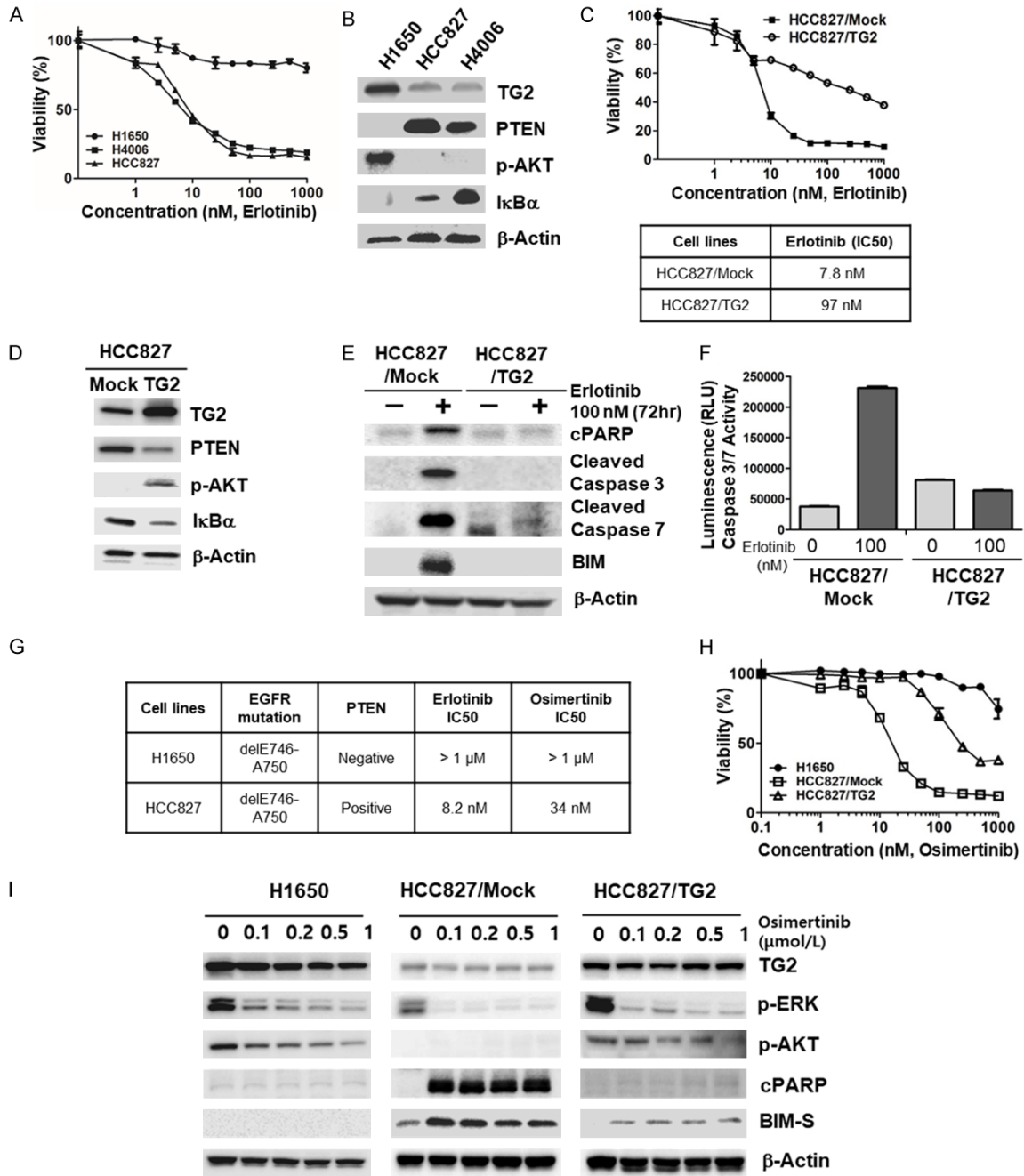


Figure 1. TG2 renders EGFR-mutated NSCLC cells resistant to EGFR-TKIs via PTEN loss and NF-κB activation in vitro. (A) H1650, H4006 and HCC827 cells were incubated in complete medium with the indicated concentration of erlotinib for 72 hours, after which cell viabilities were measured. Data are mean ± SD of triplicate measurements relative to untreated cells. (B) Western blotting analysis of TG2, PTEN, pAKT, IκBα, and β-Actin was performed in H1650, H4006 and HCC827 cells. (C) Cell viability of HCC827/Mock and HCC827/TG2 cells was measured after treatment with the indicated concentration of erlotinib for 72 hours. Data are mean ± SD of triplicate measurements relative to untreated cells. (D) Expression levels of TG2, PTEN, pAKT, IκBα, and β-Actin were determined by western blotting in HCC827/Mock and HCC827/TG2 cells. (E) After treatment of HCC827/Mock and HCC827/TG2 cells with erlotinib (100 nM) for 72 hours, the levels of PARP, cleaved caspase 3, cleaved caspase 7, BIM, and β-Actin were measured by western blotting. (F) After treatment of HCC827/Mock and HCC827/TG2 cells with erlotinib using the same conditions as in (C), caspase 3 and 7 activities were measured by the Caspase 3/7 assay kit. Data are mean ± SD of triplicate measurements. (G) IC50 value of each EGFR-TKI and mutation status in H1650 and HCC827 cells. (H) Cell viabilities were measured in H1650, HCC827, and HCC827/TG2 cells treated with osimertinib for 72 hours. Data are mean ± SD of triplicate measurements relative to untreated cells. (I) Expression levels of TG2, pERK, pAKT, cPARP (cleaved PARP), BIM-S (shortest form of BIM), and β-Actin were detected by Western blotting.

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evaluate whether TG2 is involved in the resistance to EGFR-TKIs. Initially, TG2-transfected HCC827 cell line (HCC827/TG2) overexpressed TG2, which led to NF- κ B activation by I κ B α depletion and activation of AKT (**Figure 1D**). HCC827/TG2 and H4006/TG2 cell lines displayed primary resistance to erlotinib and osimertinib, unlike parental HCC827 cells (**Figure 1C, 1G and 1H**). Further evaluation showed that after treatment with erlotinib and osimertinib, the parental HCC827 apoptotic pathway was activated with cleavage of poly (ADP-ribose) polymerase (PARP), caspase 3, and caspase 7, and expression of BIM. However, pERK and pAKT, downstream pathway of EGFR, were still activated in HCC827/TG2 and H1650 cells (**Figure 1E, 1F and 1I**) after EGFR-TKI treatment. Therefore, upregulation of TG2 might induce primary resistance to EGFR-TKIs in EGFR mutant NSCLC via NF- κ B activation by I κ B α loss and activation of AKT.

TG2 inhibition restores EGFR-TKI sensitivity in TG2-induced EGFR-TKI resistant NSCLC cells

To overcome TG2-induced resistance, we investigated whether a TG2 inhibitor can reverse or overcome the intrinsic EGFR-TKI resistance in HCC827/TG2. The combination of the TG2 inhibitor, glucosamine, with erlotinib showed to restore PTEN and I κ B α , leading to inactivation of AKT but reactivation of the apoptotic pathway (**Figure 2A**). The sensitivity to erlotinib was also restored when cells were treated with the combination (**Figure 2B**). The combination index (CI) was assessed by the Chou-Talalay method using different concentrations of the erlotinib with 1 mM glucosamine in HCC827/TG2 cells. Significant synergy (CI < 1) was observed at all concentrations tested (**Figure 2C**).

Next, we investigated whether the downregulation of TG2 in H1650 cells intrinsically resistant to EGFR-TKI therapy despite harboring activating EGFR mutation could restore the sensitivity to EGFR-TKIs. H1650 cells feature loss of PTEN due to deletions of exons 8 and 9 of the PTEN gene. As mentioned above, TG2 is overexpressed in H1650 cells and I κ B α is lost. We downregulated TG2 by transfection of TG2 siRNA and TG2 inhibitor. As a result, transfection of TG2 siRNA upregulates I κ B α (**Figure 2E**) and glucosamine also upregulate I κ B α dose-dependently (**Figure 2E**). We investigated further

whether inhibition of TG2 combined with erlotinib can overcome the primary or intrinsic resistance. When H1650 cells in which TG2 is downregulated by TG2-siRNA were treated with erlotinib, the cells line became more sensitive to erlotinib (**Figure 2H**), especially at a high dose of erlotinib. When treated with erlotinib combined with glucosamine, H1650 cells also become more sensitive to erlotinib (**Figure 2F**). Significant synergy (CI < 1) was observed at most concentrations tested (**Figure 2G**). The combination of erlotinib and glucosamine in H1650 cells upregulated I κ B α even more, compared to either glucosamine or erlotinib alone (**Figure 2I**). Interestingly, the TG2 inhibitors glucosamine and TG2 siRNA activated the apoptotic pathway or cleavage of PARP, caspase 3, caspase 7, and BIM (**Figure 2I-L**) in TG2-induced intrinsic EGFR-TKI resistant NSCLC cells, suggesting that inhibition of TG2 could overcome the intrinsic EGFR-TKI resistance. The depth of downregulation of TG2 was suggested to correlate with the sensitivity to EGFR-TKI therapy from combination of erlotinib with either siRNA inhibition or TG2 inhibitor.

Inhibition of TG2 overcomes intrinsic EGFR-TKI resistance with EGFR-TKI in xenograft models

Next, we explored whether the in vitro findings translate into in vivo results. Using mouse xenograft models of HCC827, HCC827/TG2, and H1650, we treated mice with erlotinib, glucosamine, or the combination of the two. From xenograft models of HCC827 and HCC827/TG2, we did not observe the difference in the two agents in terms of the growth. However, after treating with erlotinib, we observed the primary resistance to erlotinib in the HCC827/TG2 group (**Figure 3A, 3B**). From the xenograft model of H1650, we observed primary resistance to erlotinib. The use of glucosamine alone inhibited tumor growth and the combination of glucosamine and erlotinib additionally inhibited tumor growth of tumors in the xenograft model of H1650 (**Figure 3D, 3E**). Body weight loss and other side effects did not show prior to treatment or testing. We also observed activation of the apoptotic pathway in the xenograft models and the upregulation of proapoptotic proteins, cPARP, BIM, caspase 3, and caspase 7 with the combination treatment of erlotinib and glucosamine in both HCC827/TG2 and H1650 xenograft tumors (**Figure 3C, 3F-H**). It suggested that the TG2 inhibitor could over-

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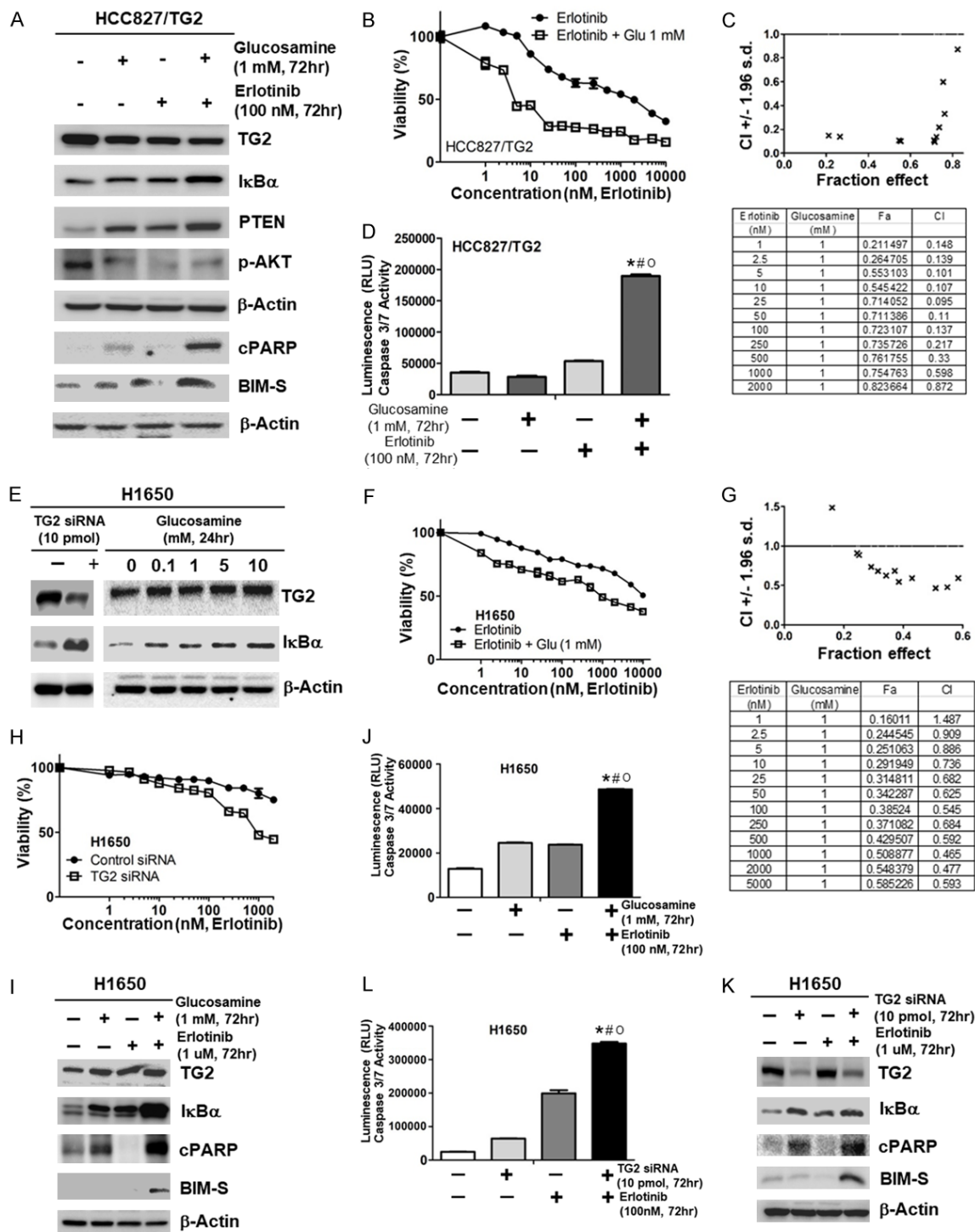


Figure 2. TG2 inhibition restores EGFR-TKI sensitivity in TG2-induced EGFR-TKI resistant NSCLC cells. (A) After treatment of HCC827/TG2 cells with or without glucosamine (1 mM), erlotinib (100 nM), or both, levels of the indicated proteins were measured by western blotting. (B) Cell viability of HCC827/TG2 cells was measured after treatment with the indicated concentration of erlotinib with or without glucosamine (1 mM) for 72 hours. Data are mean \pm SD of triplicate measurements relative to untreated cells. (C) Synergistic effect of the combination of erlotinib and glucosamine in HCC827/TG2 cells. CI denotes combination index. (D) After treatments of HCC827/TG2 cells as in (A and B), caspase 3 and 7 activities were measured using the Caspase 3/7 assay. Data are mean \pm SD of triplicate measurements. *, $P < 0.001$ combination (Combo) versus no treatment; #, $P < 0.001$ Combo versus Glucosamine alone; O, $P < 0.001$ combo versus Erlotinib alone. The mean \pm SD of three independent experiments are shown. (E) After transfection of H1650 cells with TG2 siRNA (10 pmol) or treatment of the indicated concentration of glu-

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cosamine for 24 hours, levels of TG2, I κ B α , and β -Actin were measured by western blotting. (F) Cell viability was measured after treatment of H1650 cells with erlotinib with or without glucosamine (1 mM) for 72 hours. Data are mean \pm SD of triplicate measurements relative to untreated cells. (G) Synergistic effect of the combination of erlotinib and glucosamine in H1650 cells. CI denotes combination index. (H) After transfection of H1650 cells with control siRNA or TG2 siRNA (10 pmol) for 24 hours, the indicated concentration of erlotinib was added. After 72 hours of erlotinib treatment, cell viability was measured. Data are mean \pm SD of triplicate measurements relative to untreated cells. (I) After treatment of H1650 cells with or without glucosamine (1 mM), erlotinib (1 mM), or both, levels of the indicated proteins were measured by western blotting. (J) After treatments of H1650 cells as in I, caspase 3 and 7 activities were measured using the Caspase 3/7 assay kit. Data are mean \pm SD of triplicate measurements. (K) Transfection of H1650 cells with TG2 siRNA (10 pmol), or erlotinib (1 mM), or both for 72 hours was done and the levels of the indicated proteins were measured by western blotting. (L) After transfection, drug treatment, or both as in (K), caspase 3 and 7 activities in H1650 cells were measured by the Caspase 3/7 assay kit. Data are mean \pm SD of triplicate measurements.

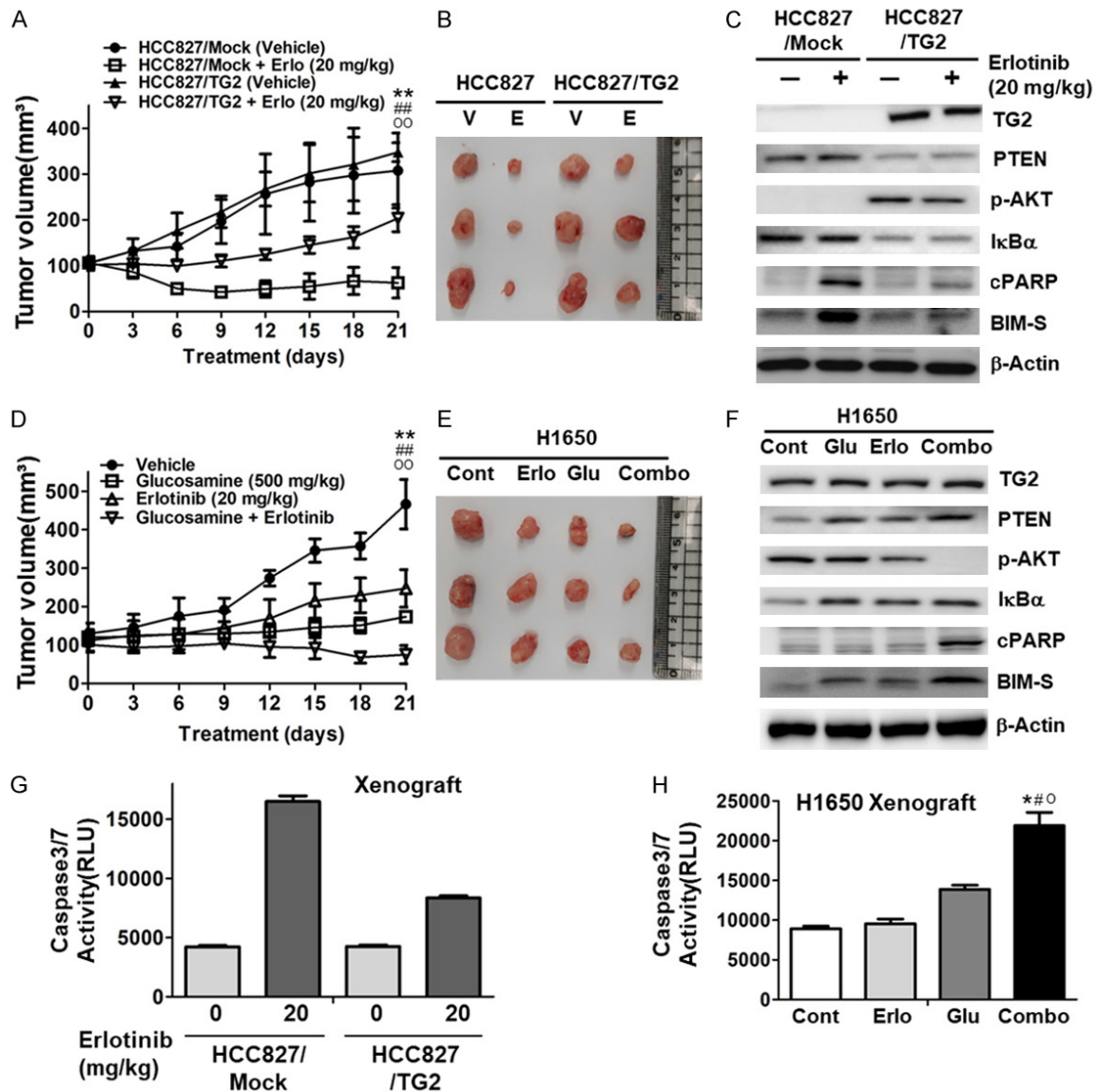


Figure 3. Inhibition of TG2 reverses the EGFR-TKI resistance in the xenograft models of EGFR mutant NSCLCs. (A and D) Nude mice were injected subcutaneously with HCC827/Mock, HCC827/TG2, and H1650 cells. HCC827/Mock and HCC827/TG2 groups were treated with vehicle (control) and erlotinib (20 mg/kg) as described in Methods. The H1650 group was treated with vehicle (control), glucosamine (500 mg/kg), erlotinib (20 mg/kg), or the combination of glucosamine and erlotinib as described in Methods. Tumor volumes were measured at the indicated times after treatments. Data are mean \pm SE for three mice per group (n=3, 2 way ANOVA with Bonferroni posttests, error bars

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represent SD). *, $P < 0.05$, **, $P < 0.01$ Combination (Combo) versus Vehicle; #, $P < 0.05$, ##, $P < 0.01$ Combo versus Glucosamine; O, $P < 0.05$, OO, $P < 0.01$ combo versus Erlotinib. The mean \pm SD of three independent experiments are shown. (B and E) Representative xenograft tumor samples of mice treated as in (A and D) showing tumors at the end of the 21-day treatment period. (C and F) Protein samples were extracted from xenograft samples of HCC827/Mock, HCC827/TG2, and H1650 as shown in (B and E). Levels of PARP, BIM, and β -Actin were measured by western blotting. (G and H) Caspase 3 and 7 activities of protein samples as in (C and F) were measured by the Caspase 3/7 assay. Data are mean \pm SD of triplicate measurements. ($n=3$, 2 way ANOVA with Bonferroni post-tests, error bars represent SD). *, $P < 0.001$ combination (Combo) versus Cont (Control); #, $P < 0.001$ Combo versus Glucosamine alone; O, $P < 0.001$ combo versus Erlotinib alone. The mean \pm SD of three independent experiments are shown.

come the EGFR-TKI resistance in EGFR mutant NSCLC.

Co-targeting EGFR and TG2 is an effective strategy for overcoming TG2-induced intrinsic resistance

The combination treatment of EGFR-TKI with caffeic acid phenethyl ester (CAPE), which blocks NF- κ B, a downstream molecule of TG2, also restored sensitivity to EGFR-TKI therapy in H1650 and HCC827/TG2 cells (**Figure 4A**). In addition, the AKT inhibitor GSK458, a downstream molecule of TG2, also recovered sensitivity to EGFR-TKI (**Figure 4B**). In addition, Although Blakely et al. suggested that NF- κ B inhibitor with EGFR-TKI restore this EGFR-TKI sensitivity and enhance patient outcomes in EGFR-TKI resistant NSCLC [15], we have tested whether our combination using TG2 inhibitor show less toxicity compare with combination using in HEK293 non-cancerous human cell line. As you can see **Figure 4C**, in similar inhibitory effect, our combination showed less toxicity than the combination with the NF- κ B inhibitor (**Figure 4D** and **4E**).

The collective data suggest the following model. In TG2-negative, PTEN-positive, EGFR-mutated NSCLC cells, EGFR-TKI is sufficient to inhibit the PI3K/AKT pathway and activate the mitogen-activated protein kinase/extracellular signal-regulated protein kinase pathway and cell proliferation (**Figure 4F**). The expression of TG2 leads to the activation of NF- κ B and PI3K/AKT signaling and loss of PTEN, thereby offsetting the impact of EGFR-TKI on cell viability (**Figure 4G**). The efficacy of EGFR inhibition can be improved dramatically by dual-inhibition of TG2, which reduces cell viability by blocking the TG2 pathway (**Figure 4H**). Our model suggests that co-targeting EGFR and TG2 may be an effective strategy for overcoming TG2-induced intrinsic resistance to EGFR-TKIs in TG2-positive, EGFR-mutated NSCLC patients.

Discussion

During the last two decades, identification of “driver mutation” and “actionable alteration” has changed a paradigm of cancer treatment and led to era of molecular-targeted therapy or precision medicine. The discovery of EGFR mutations has led to improve EGFR-TKI therapy, which has resulted in dramatic responses and longer survival outcomes in patients harboring EGFR sensitizing mutations. However, the initial hope of EGFR-TKI therapy did not last long because of the outbreak of primary or acquired resistance. Most resistant NSCLC patients with EGFR mutations inevitably succumb to their disease due to progression after EGFR-TKI treatment. Even after the introduction of 3rd generation EGFR-TKIs, which seem better than prior EGFR-TKIs, we still have to face the same adversity. Overcoming the resistance regardless of whether it is primary or acquired, is the big challenge. Fortunately, some mechanisms are involving in the acquired resistance are already known. Among them, secondary mutations, such as the T790M or C797S mutation, can or could be overcome by a new generation EGFR-TKIs. However, some but not all of patients might benefit from the newer EGFR TKIs because there are still unknown mechanisms to be elucidated for overcoming this resistance.

TG2 is a multi-functional protein with over 130 substrates and its pleiotropic functionality includes signal transduction, apoptosis, cell adhesion, cell migration, and extracellular matrix formation. TG2 is also upregulated in a number of cancers, in which the upregulation of TG2 confers resistance to chemotherapeutic agents. However, its downregulation increases the sensitivity to the agents. TG2 activates pro-survival factors, including NF- κ B and focal adhesion kinase, and inhibits the tumor suppressor, PTEN, leading to the activation of the apoptotic pathway.

TG2 induces intrinsic EGFR-TKI resistance

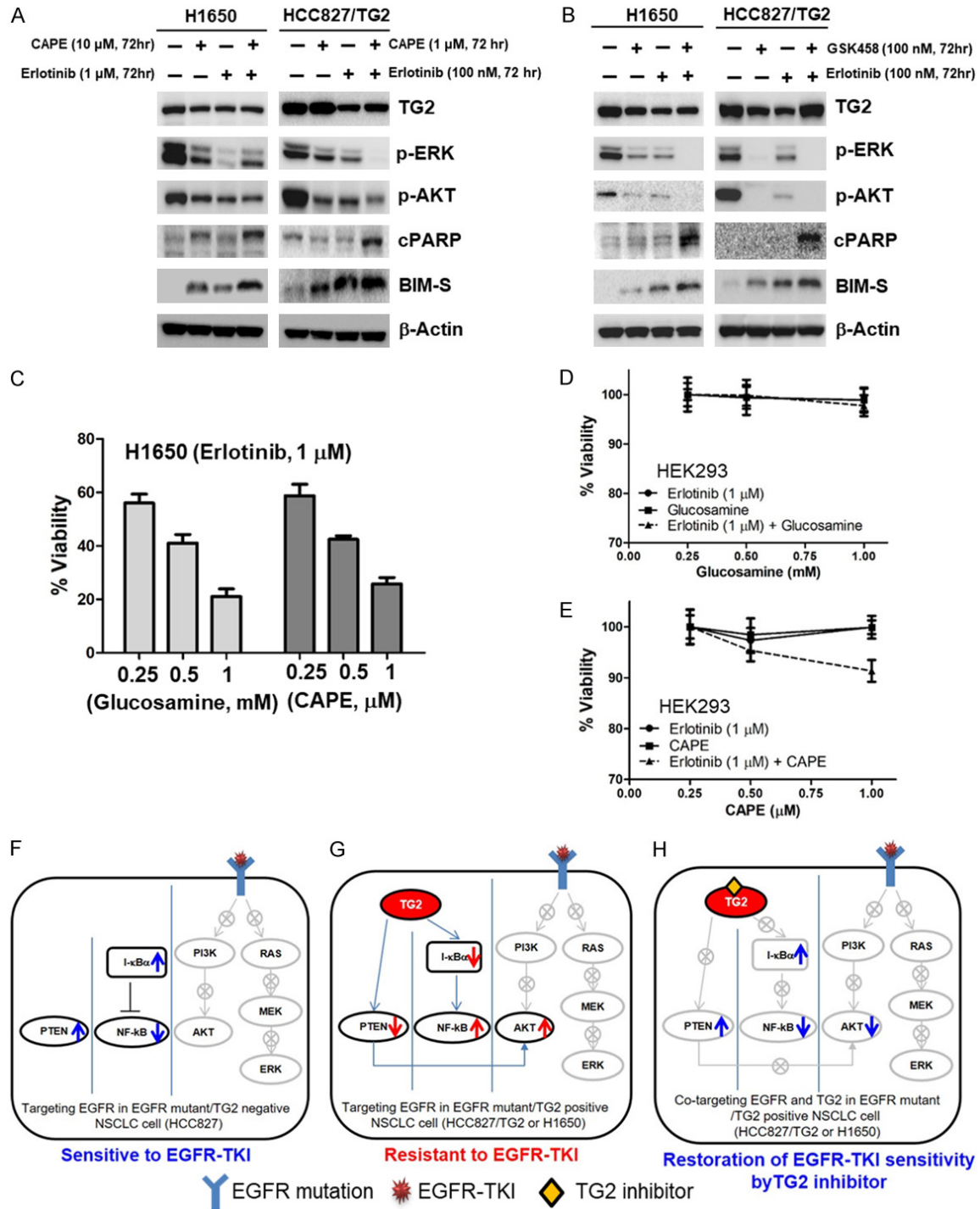


Figure 4. High TG2 expression correlates with intrinsic EGFR-TKI resistance in EGFR-mutated NSCLC patients. A. Treatments of H1650 and HCC827/TG2 cells with erlotinib (100 nM) with or without caffeic acid phenethyl ester (CAPE; NF- κ B inhibitor, H1650: 10 μ M, HCC827/TG2: 1 μ M) or combination of erlotinib and CAPE for 72 hours. Levels of the indicated proteins were measured by western blotting. B. After treatments of GSK458 (100 nM) or erlotinib (100 nM) or both drugs on H1650 and HCC827/TG2 cell lines for 72 hours, levels of the indicated proteins were measured by western blotting. C. The similar anti-cancer effect in both combination treatments; EGFR-TKI plus TG2 inhibitor vs. EGFR-TKI plus NF- κ B inhibitor. D and E. The different cytotoxicity of both combinations in normal cell, HEK293. F. Sensitive to EGFR-TKI: Targeting EGFR in EGFR mutant/TG2 negative NSCLC cell (HCC827); G. Resistant to EGFR-TKI: Targeting EGFR in EGFR mutant/TG2 positive NSCLC cell (HCC827/TG2 or H1650); H. Restoration of EGFR-TKI sensitivity by TG2 inhibitor: Co-targeting EGFR and TG2 in EGFR mutant/TG2 positive NSCLC cells (HCC827/TG2 or H1650).

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In addition, a retrospective clinical study showed interesting findings that out of 55 patients with EGFR sensitizing mutations, 19 (34.5%) patients showed high TG2 expression and the patients having high TG2 seemed to have poor survival outcomes [51]. Although the study had unavoidable limitations because it was small sample sized retrospective study and included very heterogeneous population, it suggested the possibility that TG2 might involve in the resistance against EGFR-TKI therapy ([Supplementary Figure 1](#) and [Supplementary Table 1](#)).

Those findings made us evaluate role of TG2 in tumors harboring EGFR sensitizing mutations, hoping to develop strategies overcoming the troublesome resistance during EGFR-TKI therapy. As a result, we identified that TG2 overexpression involves in the primary resistance against EGFR TKI therapy and found the possibility of developing a new combination of TG2 inhibitor and EGFR TKI in TG2 overexpressed EGFR sensitizing mutation positive tumors. We used glucosamine as TG2 inhibitor for this study because glucosamine has potent benefit, low toxicity, in vitro and in vivo, compare with others TG2 inhibitors [52]. TG2 has various functions, which might make us difficult to develop a new TG2 blocking agent. In this regard, before the development of novel specific TG2 inhibitor, we also have evaluated whether blocking downstream pathways of TG2 might be effective or not to overcome this resistance.

As we mention result part, the combination treatment of EGFR-TKI with CAPE, which blocks NF- κ B, a downstream molecule of TG2, also restored sensitivity to EGFR-TKI therapy in H1650 and HCC827/TG2 cells (**Figure 4A, 4B**). In addition, the AKT inhibitor GSK458, another downstream molecule of TG2, also recovered sensitivity to EGFR-TKI (**Figure 4A, 4B**). In addition, the combination treatment of TG2 inhibitor and EGFR-TKI is less toxicity than combination of NF- κ B inhibitor and EGFR-TKI in similar dose effect of anti-cancer effect (**Figure 4C-E**). The findings indicate that primary resistance developed by upregulated TG2 can be overcome by use of inhibitors of NF- κ B, or PI3K/AKT. TG2, which is upstream molecule of NF- κ B and PI3K/AKT pathways, inhibition could be more effective, of course. As a result, our combination treatment (EGFR-TKI and TG2 inhibitor) could be better than others (EGFR-TKI and NF- κ B inhibitor), Blakely et al. suggested, in clinic.

Interestingly, blocking TG2 or NF- κ B or AKT in H1650 cell lines devoid of PTEN was also effective, meaning that PTEN is not a pathway or, at least, a single pathway involving resistance. Therefore, the blocking upstream signal, such as TG2, might be better than blocking downstream signals, like NF- κ B or PI3K/AKT, because many pathways involve in the resistance or main pathway are not easily identified in individual patients.

To test the clinical significance of the findings, we analyzed TG2 expression by immunohistochemistry in ten formalin-fixed paraffin-embedded EGFR-mutated NSCLC specimens using TG2-specific antibody. The patients comprised eight patient who was sensitive (define as partial response or complete response), and two patients who were intrinsically resistant (define as progressive disease or stable disease) to erlotinib or gefitinib treatment ([Supplementary Table 1](#)). TG2 expression was significantly higher in the two intrinsically resistant patients than the sensitive patients ([Supplementary Figure 1](#)). These data suggest that TG2 overexpression is associated with intrinsic erlotinib or gefitinib resistance in EGFR-mutated NSCLC patients.

In the future, we have to evaluate the clinical meaning of TG2 in this clinical setting. According to previous study [51], high TG2 patients reportedly accounted for about 35%. However, the criteria is rather arbitrary. Therefore, the meaningful definition or criteria of TG2 overexpression should be determined as the efficacy of TG2 inhibition might depend on level of TG2 expression.

In addition, the present findings advocate the concept of “oncogenic addiction and oncogenic shock” [53]. According to this concept, acute inactivation of oncoprotein or a state of oncogenic shock results in rapid attenuation of oncoprotein-generated pro-survival signals, whereas oncoprotein-induced pro-apoptotic signals linger for the commitment of cancer cells to apoptotic death. However, if there are problems in pro-apoptotic signal pathway, oncogenic shock might not suffice to kill the cancer cells. TG2 is upregulated by hypoxia and other factors, attenuates the pro-apoptotic signals, and induces the resistance to EGFR-TKI therapy and perhaps other targeted agents. In such a situation, we can consider the combinations of

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agents, such as TG2 inhibitor or NF- κ B inhibitor, which are capable of activating pro-apoptotic signals for commitment to apoptotic death. To optimize EGFR-TKI therapy, we might have to test TG2 or NF- κ B or BIM status and, according to the results, consider the agents to use in combination.

In conclusion, upregulation of TG2 is involved in the resistance to EGFR-TKI therapy regardless of the type of EGFR-TKI. TG2 inhibition restores sensitivity to the EGFR-TKI therapy. Inhibition of downstream signals of TG2, such as NF- κ B inhibitor and AKT inhibitor, also enhances the efficacy of the EGFR-TKI therapy. Further clinical trials of combination of either TG2 inhibitor or NF- κ B inhibitor with EGFR-TKI therapy could be considered in patients if their tumors showed upregulated or activated TG2 regardless of whether the resistance is primary or acquired. In addition, our combination treatment with TG2 inhibitor showed clinically less toxicity than the combination with the NF- κ B inhibitor in similar anti-cancer effect.

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Disclosure of conflict of interest

Dae Ho Lee declares honoraria from Astra-Zeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, CJ Healthcare, ChongKunDang, Eli Lilly, Janssen, Merck, MSD, Mundipharma, Novartis, Ono, Pfizer, Roche, Samyang Biopharm and ST Cube. The other authors declare that they have no conflict of interest.

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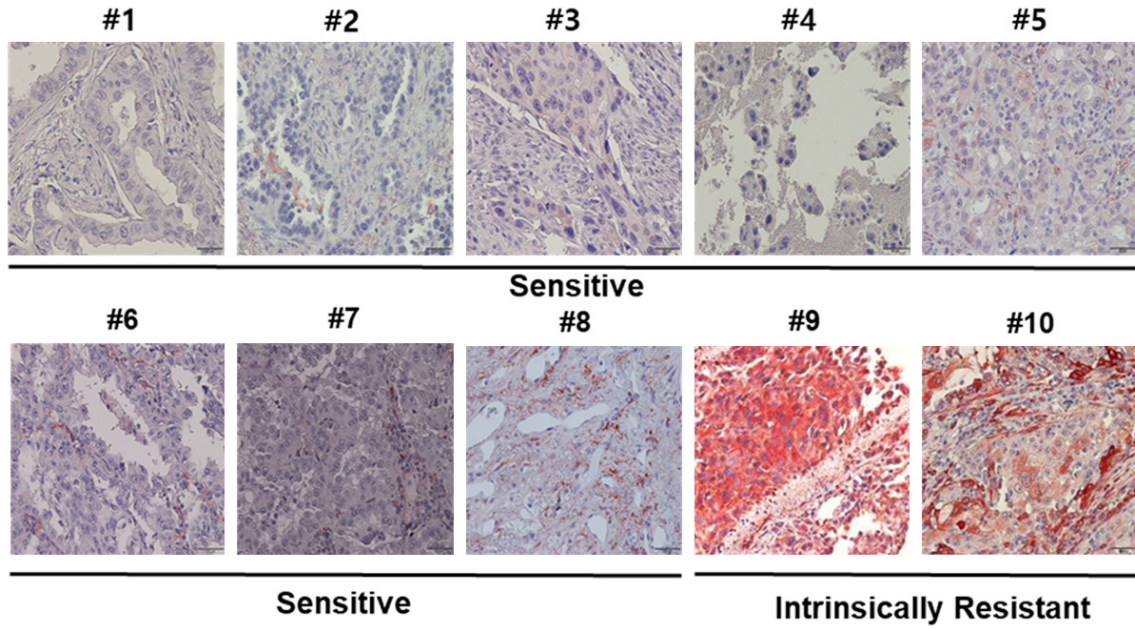
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Supplementary Figure 1. Representative immunohistochemistry results of TG2 in EGFR mutant NSCLC patients who displayed sensitivity (tissue no: #1~#8) or intrinsic resistance (tissue no: #9, #10) to erlotinib or gefitinib. Schemas explaining the mechanism of TG2-mediated intrinsic EGFR-TKI resistance. Refer to [Supplementary Table 1](#).

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Supplementary Table 1. Patient characteristics

Slide No.	Status	OS (months)	Smoking history	ECOG performance status at initiation of EGFR-TKI	Histology	Previous chemotherapies	EGFR-TKI	Response to EGFR-TKI	PFS (months)	Mutation
#1	Death	74.2	Never smoker	0	Adenocarcinoma	Yes (adjuvant NP within 6 months)	Gefitinib	Long term responder (PR)	49.8	G719X
#2	Alive	87.7	Never smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (PR)	70.1	L858R
#3	Death	84.1	Never smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (CR)	66.1	L858R
#4	Death	62.5	Never smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (PR)	61.9	L858R
#5	Alive	80.4	Never smoker	0	Adenocarcinoma	None	Gefitinib followed by Erlotinib	Long term responder (CR)	79.5	Exon 19 del
#6	Death	68.5	Never smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (PR)	49.3	Exon 19 del
#7	Alive	89.4	Never smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (PR)	68.5	Exon 19 del
#8	Alive	91.5	Ex-smoker	0	Adenocarcinoma	None	Gefitinib	Long term responder (PR)	44.3	Exon 19 del
#9	Death	6.8	Never smoker	1	Adenocarcinoma	None	Gefitinib	innate non-responder (SD)	3.6	L858R
#10	Death	3.4	Never smoker	1	Adenocarcinoma	None	Gefitinib	innate non-responder (PD)	1	L861Q