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EGFR-independent autophagy induction with gefitinib and enhancement of its cytotoxic effect by targeting autophagy with clarithromycin in non-small cell lung cancer cells

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ABSTRACT

Gefitinib (GEF), an inhibitor for EGFR tyrosine kinase, potently induces autophagy in non-small cell lung cancer (NSCLC) cell lines such as PC-9 cells expressing constitutively activated EGFR kinase by *EGFR* gene mutation as well as A549 and H226 cells with wild-type EGFR. Unexpectedly, GEF-induced autophagy was also observed in non-NSCLC cells such as murine embryonic fibroblasts (MEF) and leukemia cell lines K562 and HL-60 without EGFR expression. Knockout of *EGFR* gene in A549 cells by CRISPR/Cas9 system still exhibited autophagy induction after treatment with GEF, indicating that the autophagy induction by GEF is not mediated through inhibiting EGFR kinase activity. Combined treatment with GEF and clarithromycin (CAM), a macrolide antibiotic having the effect of inhibiting autophagy flux, enhances the cytotoxic effect in NSCLC cell lines, although treatment with CAM alone exhibits no cytotoxicity. GEF treatment induced up-regulation of endoplasmic reticulum (ER)-stress related genes such as *CHOP*/*GADD153* and *GRP78*. Knockdown of *CHOP* in PC-9 cells and *Chop*-knockout MEF both exhibited less sensitivity to GEF than controls. Addition of CAM in culture medium resulted in further pronounced GEF-induced ER stress loading, while CAM alone exhibited no effect. These data suggest that GEF-induced autophagy functions as cytoprotective and indicates the potential therapeutic possibility of using CAM for GEF therapy. Furthermore, it is suggested that the intracellular signaling for autophagy initiation in response to GEF can be completely dissociated from EGFR, but unknown target molecule(s) of GEF for autophagy induction might exist.

1. Introduction

Lung cancer has become the most common cause of death from cancer, accounting for approximately 23% of all cancer-related deaths in the world. In particular, non-small cell lung cancer (NSCLC) accounts for about nine out of ten cases of all lung cancers [1]. Small molecules for tyrosine kinase inhibitor (TKI) of epidermal growth factor receptor (EGFR) such as gefitinib (GEF) and erlotinib have become an effective therapy of choice for NSCLC patients having EGFR-mutation [2,3]. Despite the initial response to EGFR-TKIs, most patients develop resistance, often due to the emergence of a secondary mutation such as T790M, and eventually relapse. The median survival of relapsed patients is reported to be five to eight months at present [4]. Thus, a novel therapeutic strategy for both aiming the cure at initial treatment, when the NSCLC still has sensitivity to TKIs, and overcoming the resistance to a secondary mutation appears to be urgent for improving therapeutic outcomes of NSCLC.

Autophagy, a highly conserved and regulated cellular process employed by living cells to degrade proteins and organelles, has been reported to be induced after treatment with EGFR-TKIs in NSCLC and breast cancer cell lines [5,6]. Recent papers have suggested that the induction of autophagy is indispensable for the cytotoxic effect of EGFR-TKI in primary and resistant cells with mutant EGFR-in NSCLC cells [7,8], while others have reported that autophagy functions as cytoprotective as a response to metabolic

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stress [5,6,9–11]. Thus, it remains controversial whether autophagy is associated with sensitivity or resistance to EGFR-TKIs. The most recent report demonstrated that the accumulating inactivated EGFR at endosome is critical for autophagy initiation via formation of a complex with the autophagy inhibitor Rubicon in tumor cells [12]. Association of inactivated EGFR with Rubicon in turn disassociates Beclin 1 from Rubicon for autophagy initiation, which is suggested to control tumor cell metabolism and promote tumor cell survival [12].

We and others have reported that macrolide antibiotics including clarithromycin (CAM) and azithromycin, both of which are clinically well used antibiotics, have the effect of blocking autophagy flux [13–15]. We have also reported that bortezomib, a proteasome inhibitor used for the treatment of patients with multiple myeloma, induces autophagy in myeloma cell lines in vitro [16]. Simultaneous inhibition of intracellular proteolytic processes such as an ubiquitin-proteasome system with bortezomib and an autophagy-lysosome system with CAM resulted in enhancement of the apoptosis induction in myeloma cells along with accumulation of intracellular polyubiquitinated proteins and aggresome formation [15,17]. Under the concomitant inhibition of two major intracellular protein degradation systems, ER-tress loading was pronounced, probably as a result of suppression of ERAD (ER associated degradation) of ubiquitinated unfolded proteins. This leads to ER-stress mediated apoptosis induction in myeloma and breast cancer cells via up-regulation of CHOP (GADD153), a pro-apoptotic-transcription factor [16–20]. In this context, ER-stress loading appears to play a considerable role in apoptosis induction in some cancer cells [21–23].

It is of interest that TKIs for BCR-ABL including imatinib and dasatinib have been reported to induce autophagy in chronic myeloid leukemia (CML) cells along with ER-stress [24,25]. CAM-enhanced dasatinib-induced cell death in CML cells in vitro by inhibiting late stage autophagy has also been reported [26]. In several patients with resistant CML, oral administration of CAM (500 mg, b.i.d.) was suggested to potentiate TKI-treatment, although the molecular mechanism remains to be clarified [27].

Based on these lines of evidence, we hypothesized that CAM could enhance the cytotoxic effect of GEF along with ER-stress loading in NSCLC. In the present study, we examined the combined effect of GEF plus CAM as well as the biological roles of GEF-induced autophagy in NSCLC cells by using CAM as an autophagy inhibitor.

2. Material and methods

2.1. Reagents

Gefitinib (GEF) purchased from Cayman Chemical (Ann Arbor, MI) was dissolved in dimethyl sulfoxide to make stock solutions at concentrations of 10 mM. Clarithromycin (CAM) purchased from Tokyo Chemical Industry (Tokyo, Japan) was dissolved in ethanol to prepare stock solutions of 5 mg/ml. Lysosomal inhibitors E-64d and pepstatin A were obtained from Peptide Institute (Osaka, Japan). Doxycycline hydrochloride was purchased from Wako Pure Chemicals Industries (Tokyo).

2.2. Cell lines and culture conditions

For this study, NSCLC cell lines such as PC-9 were obtained from the RIKEN Bio Resource Center (Tsukuba, Japan), and A549 and H226 were from the American Type Culture Collection (ATCC) (Manassas, VA). Leukemia cell lines HL-60 and K562, along with a *Chop*$^{-/-}$ murine embryonic fibroblast (MEF) cell line (CHOP-KO-DR) established from a 13.5-day-old *Chop*$^{-/-}$ mouse embryo by SV-40 immortalization and a *Chop*$^{+/+}$ MEF cell line (DR-wild-type) established by SV-40 immortalization as a control cell line for CHOP-KO-DR, were also obtained from the ATCC. These authorized cell lines were expanded and frozen in aliquots within one month after they were obtained from the cell banks. Each aliquot was thawed and the cells were used for the experiments within two months after thawing. The NSCLC and leukemia cell lines were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Wako). CHOP-KO-DR and DR-wild-type cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The m5-7 cell line, an *Atg5* Tet-off MEF system, was a kind gift from Dr. Noboru Mizushima (Tokyo University, Graduate School and Faculty of Medicine, Tokyo, Japan). The culture conditions for knockout of the *Atg5* gene for inhibiting autophagy were previously described in detail [28]. All cell lines were cultured in a humidified incubator containing 5% CO_2 and 95% air at 37 °C.

2.3. Assessment of viable number of cells

The number of viable cells was assessed using CellTiter Blue, a cell viability assay kit (Promega, Madison, WI), according to the manufacturer's instructions as previously described in detail [15].

2.4. Immunoblotting

Immunoblotting was performed as previously described [15]. In brief, cells were lysed with RIPA lysis buffer (Nacalai Tesque, Kyoto, Japan) supplemented with a protease and phosphatase inhibitor cocktail (Nacalai Tesque). Cellular proteins were separated by SDS-PAGE and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). The membranes were probed with primary antibodies (Abs): anti-LC3B Ab (Novus Biologicals, Littleton, CO), anti-p62 (D-3) monoclonal (m) Ab, anti-GAPDH (6C5) mAb, anti-EGFR (1005) Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-cleaved caspase-3 Ab, anti-PARP Ab (Cell Signaling Technologies, Danvers, MA). Specific Abs against tyrosine phosphorylated proteins, such as anti-phosphotyrosine (4G10) mAb, were purchased from Millipore, and anti-phospho-EGFR (Tyr1173) Ab was obtained from Santa Cruz Biotechnology. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary Abs (Cell Signaling Technologies) and an enhanced chemiluminescence reagent (Millipore). Densitometry was performed using a Molecular Imager, ChemiDoc XRS System (Bio-Rad).

2.5. Gene expression analysis

A real-time polymerase chain reaction (PCR) for gene expression analysis was performed as we previously described in detail [15].

2.6. Knockout of ERGR gene by CRISPR/Cas9 –mediated genome editing and gene silence of CHOP by siRNA

Target sequences for CRISPR interference [29] were designed at CRISPR direct (http://crispr.dbcls.jp/), provided by the Database Center for Life Science (Chiba, Japan). The target sequences for human EGFR are GGAGCAGCGATGCGACCCTC (Exon 1) or TGCAAATAAAACCGGACTGA (Exon 3). Two complementary oligonucleotides with BpiI restriction sites for guide RNAs (gRNAs) were synthesized at Eurofins Genomics (Tokyo, Japan), and cloned into pX459 CRISPR/Cas9-Puro vector (Addgene, Cambridge, MA) deposited by the Feng Zhang Lab.

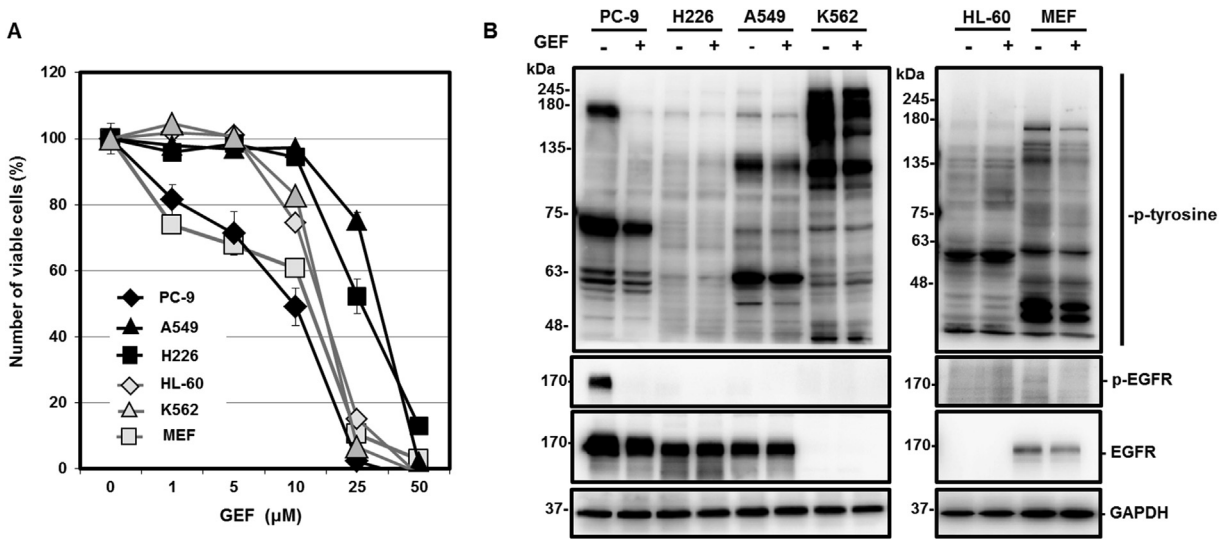

Fig. 1. Cell-growth inhibition and changes of intracellular protein tyrosine phosphorylation pattern after treatment with GEF. (A) All cell lines were treated with GEF at various concentrations for 48 h. The viable cell number was assessed by CellTiter Blue as described in Materials and methods. (B) Cells were treated with/without 5 μM of GEF for 12 h. Cells were lysed, and cellular proteins were separated by 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb, anti-EGFR Ab, and anti-phospho-EGFR (Tyr1173) Ab. Immunoblotting with anti-GAPDH mAb was performed as an internal control.

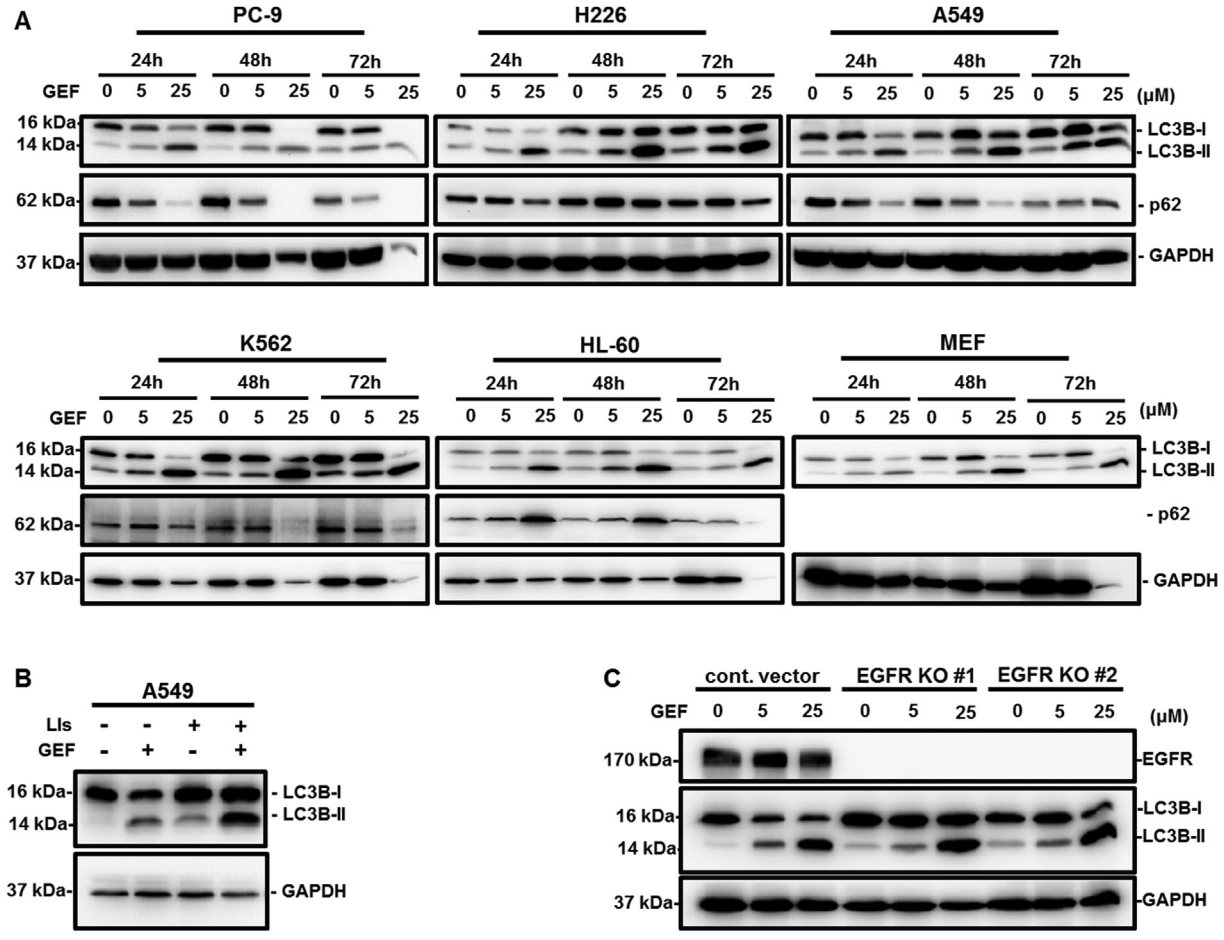

Fig. 2. Autophagy induction after treatment with GEF. (A) Cells were treated with GEF at 5 and 25 μM for 24 h–72 h. Cellular proteins were separated by 15% SDS-PAGE for LC3B and 11.25% SDS-PAGE for p62. Immunoblottings were performed using anti-LC3B Ab and anti-p62 mAb. Immunoblottings with anti-GAPDH mAb were performed as an internal control. Because of the high cytotoxicity of GEF in some cell lines, it was difficult to load the same amount of protein after 72 h exposure to GEF. Anti-p62 (D-3) mAb used in these experiments reacts with human p62 but not with murine origin. (B) A549 cells were cultured with/without GEF (25 μM) in the presence or absence of lysosomal inhibitors (LIs), E-64d (10 μg/ml) and pepstatin A (10 μg/ml) for 24 h. Cellular proteins were separated by 15% SDS-PAGE and immunoblotted with anti-LC3B Ab. (C) Two clones of A549 with *EGFR* gene knocked out by the CRISPR/Cas9 method were treated with GEF at 5 and 25 μM for 72 h. Immunoblottings were performed using anti-EGFR Ab and LC3B Ab.

A549 cells were transfected with pX459-gRNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Beginning the day after transfection, these cells were treated with 2 μg/ml of puromycin for two days. Surviving cells were reseeded at 0.5 cells per well of a 96-well plate. Expressions of EGFR in the expanded colonies were detected by immunoblotting using anti-EGFR (1005) Ab to select the *EGFR*-depleted colonies. The genome sequences of the edited locus in selected colonies were confirmed by sequence analyses performed at Eurofins Genomics.

Gene silence of *CHOP* in PC-9 cells by siRNA was performed as previously described in detail [20].

2.7. Statistical analysis

All data are expressed as mean ± SD. The statistical analysis was performed using Mann–Whitney's U test (two-tailed).

3. Results and discussion

3.1. Cell growth inhibition in NSCLC and other cell lines after treatment with GEF

As seen in Fig. 1A, GEF inhibited cell growth in NSCLC cell lines tested in a dose-dependent manner: PC-9 cells with EGFR mutation exhibited the most potent cell-growth inhibition in response to GEF as compared with A549 and H226 cells, which are the cell lines expressed with wild-type EGFR [30]. IC_{50} (50% inhibitory concentration) after 48 h-exposure to GEF was 10.4 μM in PC-9 but was 33.4 μM in A459 and 26.0 μM in H226 cells. This was well correlated with the clinical response, demonstrating that sensitizing EGFR mutations are the most predictive factor for clinical benefit with EGFR TKIs [2,3]. The higher sensitivity of PC-9 cells to GEF can be explained by the fact that they at least in part depend on the cell growth and/or survival signals produced by ligand-independent activation of mutant EGFR kinase. This is supported by our data in Fig. 1B showing that, in contrast to A549 and H226 cells, constitutive activated EGFR was observed in PC-9 cells, and that the tyrosine phosphorylation of EGFR disappeared in response to GEF.

However, GEF exhibits considerable cell-growth inhibition in MEF and non-EGFR expressing cell lines such as K562 (a CML cell line with activated BCR-ABL kinase) and HL-60 cells (an AML cell line) (Fig. 1A). (IC_{50}s for these cell lines were 13.1 μM in MEF, 16.4 μM in K562, and 16.2 μM in HL-60.)

3.2. GEF induces autophagy in an EGFR-independent manner

Treating the cells with GEF resulted in an increased expression ratio of LC3B-II to LC3B-I, which is a hallmark of autophagosome formation by the conversion of cytosolic LC3B-I into lipidated LC3B-II associated with autophagosome membrane in NSCLC cell lines (Fig. 2A) [31]. P62, a substrate of autophagy, decreased in response to GEF treatment. In addition, in the presence of lysosomal inhibitors (LIs) for blocking autophagy flux, accumulation of GEF-induced LC3B-II isoform was further enhanced as compared to treatment with either GEF or LIs (Fig. 2A, B). All of these data indicate that GEF induces autophagy in NSCLC cell lines as previously reported by others [5,8,9]. Strikingly, autophagy induction in

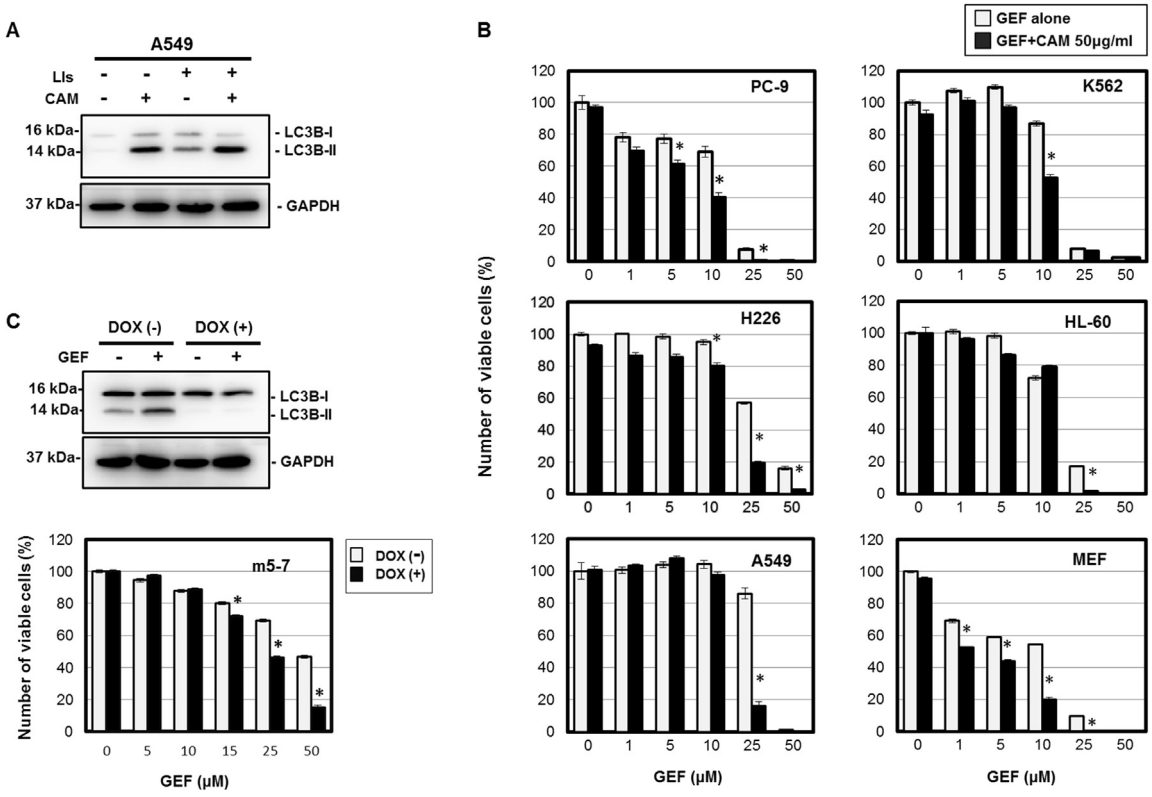

Fig. 3. CAM enhances the cytotoxic effect of GEF. (A) Autophagy flux analysis in A549 cells: Cells were treated with CAM at 50 μg/ml in the presence or absence of LIs for 48 h as described in Fig. 2B. Cellular proteins were separated by 15% SDS-PAGE and immunoblotted with anti-LC3B Ab. Immunoblotting with anti-GAPDH mAb was performed as an internal control for protein loading. (B) Cells were cultured with GEF at various concentrations for 48 h in the presence or absence of CAM (50 μg/ml). The viable cell number was assessed as described in Materials and methods. (*$p < 0.05$: CAM (−) vs. CAM (+)) (C) For complete inhibition of GEF-induced autophagy, a *Atg5* Tet-off MEF cell line, m5-7, was used [28]. After pre-treatment with/without doxycycline (Dox: 10 ng/ml) for 4 days, m5-7 cells were further cultured in the presence or absence of GEF (25 μM) for 48 h. Assessment of the viable cell number and immunoblotting with anti-LC3B Ab were performed as described above. (*$p < 0.05$: Dox (−) vs. Dox (+)).

response to GEF was still observed in non-lung cancer cell lines such as MEF, K562, and HL-60 cells. Since K562 and HL-60 are leukemia cell lines derived from a mesoderm, they do not express EGFR (Fig. 1B). Therefore, autophagy initiation by GEF appeared not to be mediated through the inhibition of EGFR kinase activity. To confirm this, we knocked out EGFR with a CRISPR/Cas9 system in A549 cells and examined whether autophagy still could be induced by GEF treatment. As seen in Fig. 2C, A549-*EGFR*-KO clones (#1 and #2) exhibited autophagy induction in response to GEF as well as parental and control clones. Thus, the underlying molecular mechanism for autophagy initiation is dissociated from EGFR kinase inactivation and even from EGFR expression.

3.3. CAM enhances GEF-induced cytotoxicity in NSCLC

In contrast to GEF treatment, autophagy flux analysis revealed that CAM treatment did not increase the LC3B-II isoform any further as compared to either treatment with LIs or CAM plus LIs in A549 cells (Fig. 3A), indicating that CAM inhibits autophagy flux in A549 cells as previously reported in myeloma and breast cancer cells [15,20]. Next, we added CAM into the culture medium for blocking GEF-induced autophagy. As seen in Fig. 3B, combined treatment with GEF and CAM at 50 μg/ml efficiently created pronounced cytotoxicity in NSCLC cell lines, whereas treatment with CAM alone yielded no cytotoxicity. Notably, CAM also enhanced the cytotoxic effect of GEF in K562, HL-60, and MEF cells. To further confirm that the enhanced cytotoxicity is due to blocking of autophagy, we used a *Atg5* Tet-off MEF cell line m5-7 [28]. Pretreatment with doxycycline (Dox) resulted in complete inhibition of GEF-induced autophagy by knockout of the *Atg5* gene, which is essential for autophagy induction. Under this condition, GEF-induced cytotoxicity was significantly pronounced as compared with that in *Atg5*$^{+/+}$ MEF cells (Fig. 3C).

3.4. ER-stress loading in NSCLC after combined treatment with GEF and CAM

We next examined whether ER-stress loading is involved in the enhanced cytotoxicity. Expression of ER-stress related genes such as *CHOP* and *GRP78* was up-regulated in response to GEF treatment within 24 h. Combined treatment with GEF and CAM resulted in pronounced gene expression, whereas treatment with CAM alone produced almost no effect as compared with untreated control cells (Fig. 4A). CHOP is an ER-stress related transcription factor that up-regulates pro-apoptotic molecules such as Bim and Bax [15,21,22], so we compared the cytotoxicity between *Chop*$^{-/-}$MEF and *Chop*$^{+/+}$MEF cell lines. *Chop*$^{-/-}$MEF cells were less sensitive to GEF than the *Chop*$^{+/+}$ MEF cell line (Fig. 4B). In addition, knockdown of *CHOP* in PC-9 cells by siRNA (PC-9 *CHOP*-KD cells) resulted in attenuation of GEF-induced cytotoxicity (Fig. 4C), indicating that ER-stress-mediated CHOP induction is involved in the cytotoxic effect of GEF. However, enhanced cytotoxicity by combining CAM plus GEF was still detectable in both the *Chop*$^{-/-}$ MEF cell line and in PC-9 *CHOP*-KD cells (Fig. 4B, C). These data suggest that GEF plus CAM did increase ER-stress loading including CHOP, but other molecule(s) appeared to be involved in the pronounced cytotoxicity by GEF plus CAM.

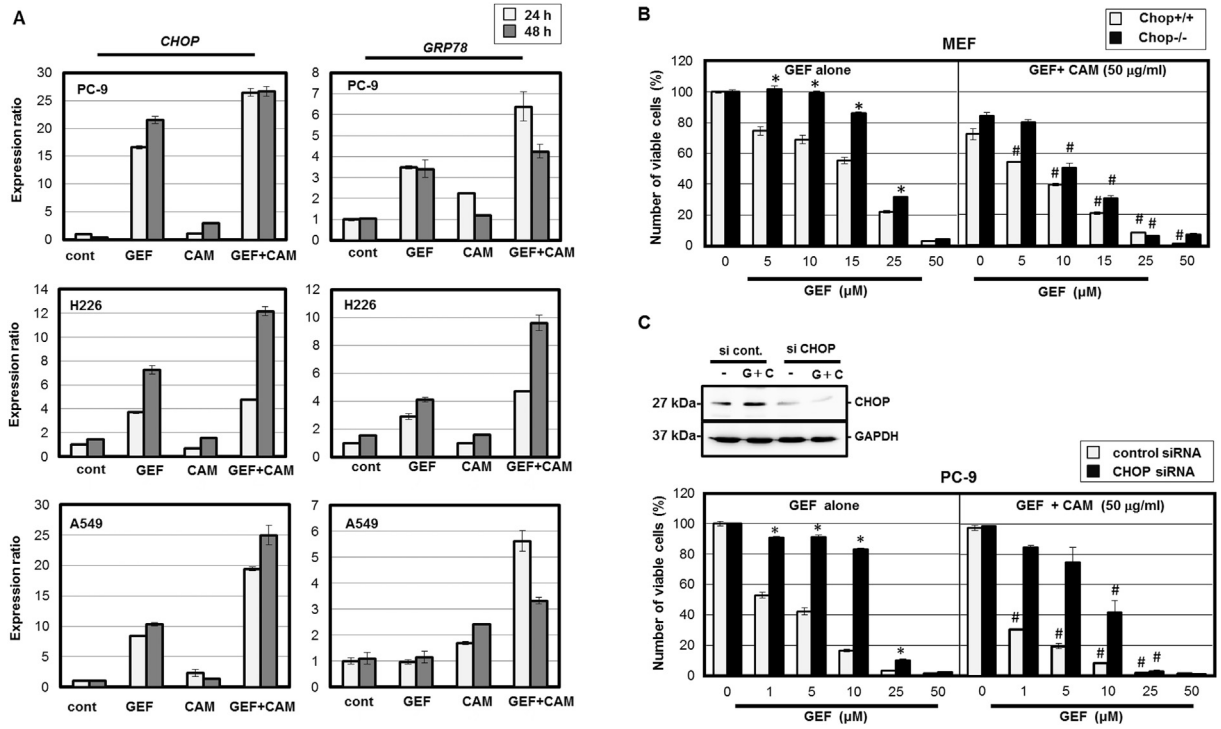

Fig. 4. ER-stress loading after treatment with GEF and/or CAM in NSCLC cell lines. (A) Kinetics of *CHOP* and *GRP78* expressions assessed by quantitative real-time PCR during 24 h and 48 h of exposure to GEF (25 μM), CAM (50 μg/ml), and GEF (25 μM) plus CAM (50 μg/ml) in NCSLC cell lines. The data of the real-time PCR products for each gene were standardized to *GAPDH* as an internal control. The expression levels of *GRP78* and *CHOP* were compared with those in untreated control cells. (B) A *Chop*$^{-/-}$MEF cell line and *Chop*$^{+/+}$MEF cell line were cultured in the presence of GEF at various concentrations with/without CAM (50 μg/ml) for 48 h. The number of viable cells was assessed by Cell TiterBlue as described in Materials and methods. (*$p < 0.05$: *Chop*$^{-/-}$MEF vs. *Chop*$^{+/+}$MEF, #$p < 0.05$: GEF alone vs. GEF + CAM) (C) PC-9 cells were treated with *CHOP* siRNA and control siRNA for 48 h. The cells were treated with/without GEF (25 μM; G) and CAM (50 μg/ml; C) for 24 h. Cellular proteins were separated by 11.25% SDS-PAGE and immunoblotted with anti-CHOP mAb. Immunoblotting with anti-GAPDH mAb was performed as an internal control for protein loading. Alternatively, after siRNA treatment, the cells were further treated with/without GEF at various concentrations in the presence or absence of CAM (50 μg/ml) for 48 h. The number of viable cells was assessed by Cell TiterBlue. (*$p < 0.05$: control siRNA vs. *CHOP* siRNA, #$p < 0.05$: GEF alone vs. GEF + CAM).

We here demonstrate that GEF induces autophagy in all cell lines regardless of EGFR expression (Fig. 2). Since all of these cell lines inhibited cell growth in response to GEF to a greater or lesser degree, autophagy might be induced as a metabolic stress response. However, comparing these cell lines, autophagy induction was not well-correlated to cell-growth inhibition but occurred to almost the same extent at 25 μM of GEF (Fig. 2A). According to our previous report, imatinib induces autophagy in various cell lines regardless of BCR-ABL expression, and, surprisingly, imatinib exhibited the cytoprotective effect in non-BCR-ABL expressing cells [32]. In the presence of imatinib, the variable cell number was significantly increased in HL-60 and MEF cells under serum deprivation [32]. These data suggest the existence of target molecule(s) of TKIs for cytoprotective autophagy initiation that might be completely dissociated from the primary target.

We here also present that CAM enhances the cytotoxicity of GEF along with blocking GEF-induced cytoprotective autophagy. Since CAM is a clinically well used macrolide antibiotic, this drug appears to be a strong candidate for TKI-therapy for improving therapeutic outcomes in NSCLC patients. Although CAM enhances the cytotoxicity of GEF along with ER-stress loading (Figs. 3 and 4), we could not clearly detect pronounced apoptosis induction as assessed by Annexin V staining nor could we detect cleavages of caspase-3 and PARP or morphological apoptotic features such as chromatin condensation, nuclear fragmentation, and formation of an apoptotic body (Supplementary data, Figs. S1 to S3.). In addition, *Chop*$^{-/-}$MEF and PC-9 *CHOP*-KD cells, both of which should be resistant against ER-stress mediated apoptosis, still exhibited pronounced cytotoxicity after combined treatment (Fig. 4B, C). Therefore, non-apoptotic cell death including necroptosis might be involved in this phenomenon [33,34]. Further studies for identifying the target molecule(s) for autophagy induction as well as identifying the true executor(s) for pronounced cell death are worthy of being carried out as the next step.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.162.

Transparency document

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