Restricting extracellular Ca\(^{2+}\) on gefitinib-resistant non-small cell lung cancer cells reverses altered epidermal growth factor-mediated Ca\(^{2+}\) response, which consequently enhances gefitinib sensitivity

Mi Seong Kim\(^{1,2}\), So Hui Kim\(^{3}\), Sei Hoon Yang\(^{4}\), Min Seuk Kim\(^{1*}\)

\(^{1}\) Department of Oral Physiology, Institute of Biomaterial-Implant, School of Dentistry, Wonkwang University, Iksan, Republic of Korea, \(^{2}\) Wonkwang Dental Research Institute, School of Dentistry, Wonkwang University, Iksan, Republic of Korea, \(^{3}\) Department of Carbon Convergence Engineering, College of Engineering, Wonkwang University, Iksan, Republic of Korea, \(^{4}\) Department of Internal Medicine, School of Medicine, Wonkwang University, Iksan, Republic of Korea

\(^{*}\) These authors contributed equally to this work.

\(^{\#}\) happy1487@wku.ac.kr

Abstract

Non-small cell lung cancer (NSCLC), one of the leading causes of cancer-related death, has a low 5-year survival rate owing to the inevitable acquired resistance toward antitumor drugs, platinum-based chemotherapy, and targeted therapy. Epidermal growth factor (EGF)-EGF receptor (EGFR) signaling activates downstream events leading to phospholipase C/inositol trisphosphate (IP\(_3\))/Ca\(^{2+}\) release from IP\(_3\)-sensitive Ca\(^{2+}\) stores to modulate cell proliferation, motility, and invasion. However, the role of EGFR-mediated Ca\(^{2+}\) signaling in acquired drug resistance is not fully understood. Here, we analyzed alterations of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) responses between gefitinib-sensitive NSCLC PC-9 cells and gefitinib-resistant NSCLC PC-9/GR cells, and we found that acute EGF treatment elicited intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) oscillations, dependent on phospholipase C and EGFR activation. Consequently, restricting [Ca\(^{2+}\)]\(_e\) in PC-9/GR cells upregulated gefitinib-mediated poly (ADP-ribose) polymerase cleavage, an increase in Bax/Bcl-2 ratio, cytotoxicity, and apoptosis. In addition, nuclear factor of activated T cell (NFAT1) induction in response to EGF was inhibited by gefitinib in PC-9 cells, whereas EGF-mediated NFAT1 induction in PC-9/GR cells was sustained regardless of gefitinib treatment. Restricting [Ca\(^{2+}\)]\(_e\) in PC-9/GR cells significantly reduced EGF-mediated NFAT1 induction. These findings indicate that spontaneous [Ca\(^{2+}\)]\(_e\) influx in NSCLC cells plays a pivotal role in developing acquired drug resistance and suggest that restricting [Ca\(^{2+}\)]\(_e\) may be a potential strategy for modulating drug-sensitivity.
Introduction

The incidence of non-small cell lung cancer (NSCLC) is steadily increasing and accounts for 85% of lung cancer subtypes. Owing to its recurrence, NSCLC has a low 5-year survival rate of <15% [1]. Since the development of first-generation epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as gefitinib, erlotinib, and erlotinib, diverse targeted therapies operating at the molecular and genetic levels have emerged rapidly. Despite these developments, intrinsic or acquired drug resistance to chemotherapeutic agents allows cancer cells to bypass cell death. Overexpression and over-activity of EGFR are observed in >60% of NSCLC cells [2]. Moreover, prolonged treatment with EGFR-TKIs frequently causes EGFR mutations and interferes with its underlying signaling pathways; thus, prolonged EGFR-TKI use limits its clinical efficacy [3].

In the 2019-guideline v3, the National Comprehensive Cancer Network indicates that genes, including EGFR, ALK, BRAF, KRAS, HER2, ROS1, RET, and MET, are therapeutic targets for treating NSCLC. TKIs targeting these fundamentally inhibit signaling cascades related to cell proliferation, by which cancer cells frequently show acquired drug resistance owing to gene mutations, including rearrangement, amplification, and point mutation [4, 5]. According to the Kyoto Encyclopedia of Genes and Genomes database, EGFR and its underlying mechanisms appear dependent on the intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) signaling pathway in NSCLC, in which EGFR activation sequentially elicits phospholipase C\textgamma (PLC\textgamma) phosphorylation, inositol trisphosphate (IP\textsubscript{3}) production, Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER), and protein kinase C activation. Acute human glioma cell stimulation with EGF evokes intracellular Ca\textsuperscript{2+} responses as oscillations, which are blocked by EGFR inhibitors [6]. Thus, the correlation between intracellular Ca\textsuperscript{2+} signaling and acquired drug resistance appears to be interactive; however, the role of Ca\textsuperscript{2+} signaling in drug resistance is not fully understood.

Free intracellular Ca\textsuperscript{2+} acts as a second messenger to regulate the proliferation, migration, and apoptosis of cancer cells [7]. Ca\textsuperscript{2+} depletion in the ER mediates Stromal interaction molecule 1 (Stim1)-dependent Ca\textsuperscript{2+} influx through store-operated Ca\textsuperscript{2+} channels (SOCCs), such as ORAI calcium release-activated calcium modulator 1 (Orai1) and (transient receptor potential channels) TRPCs [8]. Altered Ca\textsuperscript{2+} signaling in cancer development and progression has been frequently observed. Increased Ca\textsuperscript{2+} influx through TRPC5 upregulates autophagic flux to prevent cancer-cell death and promotes drug resistance [9]. Anticancer drugs, including cisplatin, 5-fluorouracil, and gemcitabine in osteosarcomas and pancreatic adenocarcinomas, appear to enhance Orai1 and Stim1 expression, which prevents drug-mediated cell death [10, 11]. Notably, treatment using EGFR-targeting afatinib causes Ca\textsuperscript{2+} signaling-related gene expression in PC-9 cells. Reduced extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]e) levels increase the sensitivity of PC-9 cells to afatinib [12]. These reports strongly suggest that Ca\textsuperscript{2+} signaling in cancer cells is significantly associated with the development of acquired drug resistance. However, the role of EGFR-mediated Ca\textsuperscript{2+} signaling in acquired drug resistance is not fully understood.

In this study, we demonstrated that the EGF-mediated Ca\textsuperscript{2+} response in NSCLC cells was altered depending on gefitinib resistance and [Ca\textsuperscript{2+}]e restriction on gefitinib-sensitive and gefitinib-resistant cells elicited identical [Ca\textsuperscript{2+}]i oscillations, which were associated with the modulation of gefitinib efficacy.

Materials and methods

Cell culture and reagents

The lung adenocarcinoma cell line, PC-9, and its gefitinib-resistant sub-cell line, PC-9/GR possessing the T790M mutation, were gifted by Dr. Jin Kyung, Rho [13]. Cells were cultured in

Competing interests: The authors have declared that no competing interests exist.
RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37°C in 5% CO2. To determine the effects of [Ca2+]i restriction, the culture medium was exchanged with RPMI-1640 without CaCl2 (MyBioSource, CA, USA) and 1 mM CaCl2 was added. Cyclopiazonic acid was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The mouse monoclonal antibodies against nuclear factor of activated T cell (NFAT1; cat # sc-7296), Orai1 (cat # sc-377281), Bcl-2 (cat # sc-509), and Bax (cat # sc-20067) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). U-compounds (U73122 and U73343) were obtained from Sigma Aldrich (St. Louis, MO, USA). Human EGF, gefitinib, and rabbit polyclonal antibodies against poly (ADP-ribose) polymerase-1 (PARP; cat #9542), stromal interaction molecule 1 (STIM1; cat #5668), sarco/endoplasmic reticulum Ca2+-ATPase 2 (SERCA2; cat #4388), and β-actin (cat #4967) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Measurement of [Ca2+]i

The [Ca2+]i was measured using a Ca2+-sensitive fluorescence dye, Fura-2/AM (Sigma Aldrich) as described previously [14]. Briefly, cells were plated on coverslips at 80% confluence and loaded with Fura-2/AM (5 μM) for 1 h at 37°C in a 5% CO2 incubator. Cells were perfused with HEPES buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 10 mmol/L HEPES, and 10 mmol/L glucose. The pH and osmolarity were adjusted to 7.4 and 310 mOsm. Ca2+-free HEPES buffer was replaced with 1 mmol/L EGTA. Following brief washing with HEPES buffer, trapped intracellular Fura-2 was excited at 340 and 380 nm. Emitted fluorescence at 510 nm was captured using a charge-coupled device camera. Images were analyzed using MetaFluor software (Molecular Devices, San Jose, CA, USA) and presented as F340/F380 ratio.

Cell viability assay

Cell viability was determined using EZ-CYTOX (Daeil Lab Service Co. Ltd., Seoul, South Korea), following the manufacturer’s procedure. In brief, cells were treated with EZ-Cytox (10 μL) in each well and incubated for 30 min. Cell viability was measured at OD 450 nm using the iMAX Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cytotoxicity assay

Cytotoxicity was determined by measuring extracellular glucose-6-phosphate dehydrogenase (G6PD) levels using a Vybrant cytotoxicity assay kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocols. Briefly, cells were seeded onto 96-well plates and incubated under indicated conditions. Cell culture medium without cells was collected and G6PD activity was determined at excitation and emission wavelengths of 544 and 590 nm, respectively. The result is expressed as the percentage of total G6PD detected in cell lysates from parallel wells.

Flow cytometry

The early apoptosis rate was determined using the FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, USA) following the manufacturer’s protocol. In brief, after a 24-h incubation, cells were washed with Cell Staining Buffer and resuspended with the Annexin V-FITC and PI mixture for 15 min at room temperature in the dark. After adding Annexin V Binding buffer, a minimum of 10,000 cells were analyzed using FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ, USA).
Western blot

Protein levels were determined using western blotting [15]. In brief, cells were lysed with RIPA buffer (Invitrogen) containing proteases and a phosphatase inhibitor cocktail (Invitrogen). Total lysates were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, separated using gel electrophoresis, transferred onto polyvinylidene fluoride membranes (0.2 μm pore size), and incubated with primary antibodies PARP (1:1000), Bax (1:1000), Bcl-2 (1:1000), and NFAT1 (1:1000); horseradish peroxidase-conjugated IgG was used as the secondary antibody. Immunoreactive proteins were detected using AzureSpot 2.0 (Azurebiosystems, CA, USA).

Statistical analysis

Statistical analysis was conducted using Origin 2020 software (OriginLab Corporation, MA, USA). Data are presented as the mean ± standard deviation of observations obtained from more than three independent experiments. Statistical differences were analyzed using one-way ANOVA followed by Tukey’s post hoc test and T-test. Values of $p < 0.05$ were considered statistically significant.

Results

Basal level of $[\text{Ca}^{2+}]_i$ in PC-9/GR cells is sustained by spontaneous extracellular $\text{Ca}^{2+}$ influx, resulting in abolishment of EGF-mediated $[\text{Ca}^{2+}]_i$ oscillations

To determine altered EGF-mediated $[\text{Ca}^{2+}]_i$ responses between PC-9 and PC-9/GR cells, we performed a ratiometric assay using Fura-2/AM. Acute treatment with 200 ng/mL of EGF induced $[\text{Ca}^{2+}]_i$ oscillations in PC-9 cells but not in PC-9/GR cells (Fig 1A). PC-9/GR cells showed a more highly sustained basal level of $[\text{Ca}^{2+}]_i$ than PC-9 cells; thus, we examined the spontaneous $\text{Ca}^{2+}$ influx in both cell types. To evaluate the spontaneous $[\text{Ca}^{2+}]_e$ influx and determine ER $\text{Ca}^{2+}$ content, cells were exposed to $\text{Ca}^{2+}$-free HEPES buffer and HEPES buffer (1 mM $\text{Ca}^{2+}$). Cells were treated with cyclopiazonic acid to deplete ER $\text{Ca}^{2+}$. The spontaneous $\text{Ca}^{2+}$ influx (indicated as F1) was greater in PC-9/GR cells than in PC-9 cells, and ER $\text{Ca}^{2+}$ content in PC-9/GR cells was 15% lower than that in PC-9 cells (Fig 1B). We additionally characterized the expression of 3 different genes, Orai1, STIM1, and SERCA2, which are essential for mediating store-operated $\text{Ca}^{2+}$ entry (SOCE). Expression of dimeric Orai1 and STIM1 showed no significant difference between PC-9 and PC-9/GR cells, whereas SERCA2 in PC-9/GR cells was significantly reduced by about 35% compared to PC-9 (S1 Fig). These results suggest that drug-resistant tumor cells somehow develop altered intracellular $\text{Ca}^{2+}$ signaling, which may act as a bypass to activate downstream signals for cell survival.

Altered EGF-mediated $[\text{Ca}^{2+}]_i$ response in PC-9/GR cells is reversed in an identical manner to that of PC-9 cells by restricting $[\text{Ca}^{2+}]_e$

Consequently, we examined the effects of $[\text{Ca}^{2+}]_e$ restriction on EGF-stimulated PC-9 and PC-9/GR cells. EGF-mediated $[\text{Ca}^{2+}]_i$ oscillations in PC-9 cells lasted even in the absence of extracellular $\text{Ca}^{2+}$ (Fig 2A). PC-9/GR cells somehow exerted $[\text{Ca}^{2+}]_i$ oscillations upon exposure to $\text{Ca}^{2+}$-free HEPES buffer, which was in a manner identical to that in PC-9 cells. Based on reports that EGF-EGFR signaling activates PLC and $[\text{Ca}^{2+}]_i$ mobilization [16], we examined whether the $[\text{Ca}^{2+}]_i$ oscillations evoked by $[\text{Ca}^{2+}]_e$ restriction were dependent on PLC and EGFR activation. Low $[\text{Ca}^{2+}]_e$-induced $[\text{Ca}^{2+}]_i$ oscillations were abolished by U73122 (2 μM), an inhibitor of PLC, and gefitinib (0.1 μM) in PC-9 and PC-9/GR cells (Fig 2B and
These results suggest that restricting \([\text{Ca}^{2+}]_e\) may revert the cytotoxic effects of gefitinib on PC-9/GR cells. \([\text{Ca}^{2+}]_e\) restriction and PLC inhibition synergistically exacerbate gefitinib-induced cytotoxicity and cell viability in PC-9/GR cells.

\([\text{Ca}^{2+}]_e\) restriction and PLC inhibition synergistically exacerbate gefitinib-induced cytotoxicity and cell viability in PC-9/GR cells.

Gefitinib reduces EGFR signaling by inhibiting the ATP-binding pocket in the EGFR kinase domain, which sequentially results in cell cycle arrest and apoptosis [17]. As PC-9 and PC-9/GR cells showed identical \([\text{Ca}^{2+}]_i\) oscillations after the removal of \([\text{Ca}^{2+}]_e\), we examined whether the low \([\text{Ca}^{2+}]_e\)-induced \([\text{Ca}^{2+}]_i\) oscillations were associated with gefitinib-induced cytotoxicity and cell viability. PC-9 and PC-9/GR cells were treated with gefitinib in a dose-dependent manner (0.1, 1, and 5 \(\mu\text{M}\)) and incubated in a culture medium supplemented with...
Fig 2. Characterizations of low \([\text{Ca}^{2+}]_e\)-mediated \([\text{Ca}^{2+}]_i\) oscillations. PC-9 and PC-9/GR cells loaded with Fura-2/AM were used to determine \([\text{Ca}^{2+}]_i\) in response to low \([\text{Ca}^{2+}]_e\), U73122, and gefitinib. (A) Induction of \([\text{Ca}^{2+}]_i\)
or without Ca\(^{2+}\). After 24- and 48-h incubation periods, cytotoxicity and cell viability, respectively, were evaluated using enzymatic assays. We first showed that gefitinib-treated PC-9 cells exhibited a significant increase in cytotoxicity and decrease in cell viability in a dose-dependent manner regardless of [Ca\(^{2+}\)]\(_e\) (left panel, Fig 3A and 3B). However, gefitinib in 1-mM [Ca\(^{2+}\)]\(_e\) had no effect on cytotoxicity and cell viability in PC-9/GR cells. Thus, [Ca\(^{2+}\)]\(_e\) restriction significantly exacerbated gefitinib-induced cytotoxicity and cell viability compared with the non-gefitinib-treated group and the group treated with gefitinib in 1-mM [Ca\(^{2+}\)]\(_e\) (mid panel, Fig 3A and 3B). We investigated the synergistic effects of PLC inhibition and [Ca\(^{2+}\)]\(_e\) restriction on cytotoxicity and cell viability. PC-9/GR cells were pretreated with U73122 and U73343 (2 \(\mu\)M), followed by treatment with gefitinib, either in the absence or presence of [Ca\(^{2+}\)]\(_e\). PLC inhibition by U73122 and [Ca\(^{2+}\)]\(_e\) restriction synergistically exacerbated cytotoxicity and cell viability (Right panel, Fig 3A and 3B).

Restricting [Ca\(^{2+}\)]\(_e\) in PC-9/GR cells enhances gefitinib-mediated early apoptosis

Biochemical assays indicated that gefitinib-mediated cytotoxicity was significantly increased by [Ca\(^{2+}\)]\(_e\) restriction in PC-9/GR cells. We investigated the effects of [Ca\(^{2+}\)]\(_e\) restriction on the activation of early apoptotic markers. To determine the apoptotic induction, whole-cell lysates were subjected to detect PARP cleavage and Bax/Bcl-2 expression. Gefitinib treatment caused the induction of apoptotic markers in PC-9 cells, irrespective of [Ca\(^{2+}\)]\(_e\) (Fig 4A). [Ca\(^{2+}\)]\(_e\) restriction in gefitinib-treated PC-9/GR cells resulted in higher PARP cleavage and Bax/Bcl-2 ratio than gefitinib-treated PC-9/GR cells in the presence of [Ca\(^{2+}\)]\(_e\). Next, we performed flow cytometry to quantify early apoptotic cells. [Ca\(^{2+}\)]\(_e\) restriction in gefitinib-insensitive cells significantly enhanced early apoptosis in the presence of gefitinib (5 \(\mu\)M) compared to cells treated with the same dose of gefitinib in the presence of [Ca\(^{2+}\)]\(_e\) (Fig 4B).

Restriction of [Ca\(^{2+}\)]\(_e\) reduces EGF-mediated NFAT1 induction in gefitinib-resistant cells

Among the isoforms of NFATs, NFAT1 expression is higher in NSCLC cells and this overexpression is related to the poor survival of patients with NSCLC [18]. This led us to examine whether EGF-stimulated tumor cells elicited NFAT1 induction and whether this was affected by [Ca\(^{2+}\)]\(_e\) restriction on gefitinib-resistant cells. Following FBS starvation for 1 h, EGF-treated (200 ng/mL) cells were incubated. EGF-mediated NFAT1 induction was significantly reduced by gefitinib (5 \(\mu\)M) in PC-9 cells (Fig 5A), whereas it was unaffected in gefitinib-treated PC-9/GR cells in the presence of [Ca\(^{2+}\)]\(_e\) (Fig 5B). [Ca\(^{2+}\)]\(_e\) restriction on gefitinib-treated PC-9/GR cells resulted in the reduction of EGF-mediated NFAT1 induction (Fig 5B). These results indicate that NFAT1 is regulated by EGFR activation and spontaneous [Ca\(^{2+}\)]\(_i\) influx is critical for inducing NFAT1 in gefitinib-resistant cells.

Discussion

Diverse signaling pathways underlying EGFR activation have been implicated in various cellular functions [16]. Increasing evidence shows that altered [Ca\(^{2+}\)]\(_i\) signaling plays a key role in tumorigenesis [19]. However, its role in acquired drug resistance is not fully understood. A
study has shown that the binding of EGF to EGFR in human glioma cells induces tyrosine kinase-dependent Ca\textsuperscript{2+} oscillations [6]. The expression of sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase and IP\textsubscript{3}R in hepatocellular carcinoma cells appears to be lower and higher, respectively, than that in human bronchial epithelial cells, leading to reduced Ca\textsuperscript{2+} content in the ER [20]. Orai3, which mediates SOCE and allows the proliferation and metastasis of abnormal cells, has been reported to be overexpressed in NSCLCs [21]. Moreover, inhibiting Stim1 and Orai1 results in the modulation of tumor migration, metastasis, and proliferation in other

Fig 3. Synergistic effects of low [Ca\textsuperscript{2+}]e and U73122 on gefitinib-mediated cytotoxicity. Gefitinib-treated cells with or without [Ca\textsuperscript{2+}]e and U73122 were incubated for 24 h and 48 h to evaluate cytotoxicity and cell viability, respectively. (A) Gefitinib-induced cytotoxicity in PC-9 and PC-9/GR cells was evaluated by measuring G6PD activity. Data are presented as proportion of released G6PD (% total; n = 4), and are given as the mean ± S.D. *P < 0.05 compared with control (only vehicle-treated in 1 mM Ca\textsuperscript{2+}) group or between indicated groups (B) Cells cultured under indicated conditions were incubated for 48 h and subjected to cell viability assay. Data are presented as relative to control (% control; n = 4) and given as mean ± S.D. *P < 0.05 compared with control (only vehicle-treated in 1 mM Ca\textsuperscript{2+}) group or between indicated groups.

https://doi.org/10.1371/journal.pone.0238155.g003
Based on these reports, we assumed that EGF-mediated Ca\(^{2+}\) signaling is altered depending on acquired drug resistance. Considering that \([\text{Ca}^{2+}]_{\text{e}}\) influx (indicated as \(F_2\) in Fig 1B) mediated by ER Ca\(^{2+}\) depletion caused no difference in both cell types in our study, increased spontaneous \([\text{Ca}^{2+}]_{\text{e}}\) influx in PC-9/GR cells appeared to be facilitated by low ER Ca\(^{2+}\) content. Thus, we showed that gefitinib-resistant cells present EGF-mediated Ca\(^{2+}\) signaling differently than gefitinib-sensitive cells. EGF-mediated \([\text{Ca}^{2+}]_{\text{i}}\) oscillations were observed only in gefitinib-sensitive cells. Moreover, basal \([\text{Ca}^{2+}]_{\text{i}}\) levels were more sustained and ER-Ca\(^{2+}\) levels were lower in PC-9/GR cells than in PC-9 cells.

PC-9 and PC-9/GR cells showed similar \([\text{Ca}^{2+}]_{\text{i}}\) oscillations following \([\text{Ca}^{2+}]_{\text{e}}\) restriction in the presence of EGF. The low \([\text{Ca}^{2+}]_{\text{e}}\)-mediated \([\text{Ca}^{2+}]_{\text{i}}\) oscillations were abolished by gefitinib even in PC-9/GR cells. In most other cell types, \([\text{Ca}^{2+}]_{\text{e}}\) influx is essential in maintaining agonist-activated \([\text{Ca}^{2+}]_{\text{i}}\) oscillations [23]. Besides maintaining \([\text{Ca}^{2+}]_{\text{i}}\) oscillations in the absence of \([\text{Ca}^{2+}]_{\text{e}}\), the acute generation of \([\text{Ca}^{2+}]_{\text{i}}\) oscillations following the restriction of \([\text{Ca}^{2+}]_{\text{e}}\) in PC-9/GR cells was unusual. Zanotti et al. reported that low \([\text{Ca}^{2+}]_{\text{e}}\) induces diverse patterns of Ca\(^{2+}\) propagation including \([\text{Ca}^{2+}]_{\text{i}}\) oscillations in glial cells [24]. In parathyroid cells, Ca\(^{2+}\)-sensing receptors detect increased levels of \([\text{Ca}^{2+}]_{\text{e}}\) and induce \([\text{Ca}^{2+}]_{\text{i}}\) signaling, which results in regulating the secretion of parathyroid hormones [25]. Herein, we first report that restricting \([\text{Ca}^{2+}]_{\text{e}}\) on EGF-treated gefitinib-resistant NSCLC cells leads to the induction of \([\text{Ca}^{2+}]_{\text{i}}\) oscillations.
oscillations, which is identical to the \([Ca^{2+}]_i\) response in gefitinib-sensitive cells. Low \([Ca^{2+}]_e\)-mediated \([Ca^{2+}]_i\) oscillations were dependent on the blockade of EGFR activation by gefitinib, even in gefitinib-resistant cells. Considering the lower ER \(Ca^{2+}\) content in PC-9/GR cells than in PC-9 cells, cellular organelles, such as lysosomes and mitochondria, might partake in maintaining \(Ca^{2+}\) oscillations. However, identifying \(Ca^{2+}\) stores that are involved in maintaining \([Ca^{2+}]_i\) oscillations requires further investigation. We used PC-9/GR cells with secondary mutations [13], which enhanced the affinity of EGFR toward ATP and rendered it gefitinib-resistant. We observed that low \([Ca^{2+}]_e\)-mediated \([Ca^{2+}]_i\) oscillations were abolished by gefitinib and U73122 in PC-9/GR and PC-9 cells, suggesting that the absence of \([Ca^{2+}]_e\) led to enhanced gefitinib sensitivity and reversed gefitinib-mediated cytotoxicity and apoptosis.

Mulder et al. demonstrated that initial targeted therapy on PC-9 cells increases the activity of \(Ca^{2+}\) signaling-related proteins and deprives the cell of extracellular \(Ca^{2+}\), which results in the marked enhancement of afatinib efficacy [12]. Our data indicated that increased \([Ca^{2+}]_e\) influx in PC-9/GR cells contributed to gefitinib resistance; therefore, restricting \([Ca^{2+}]_e\) could be key in enhancing drug sensitivity.
The induction of NFATs 1–4 is regulated by \([\text{Ca}^{2+}]_i\) increase, which is mediated by the receptor-activated PLC pathway or extracellular \(\text{Ca}^{2+}\) influx in immune cells [26]. Inactive NFATs exist in hyper-phosphorylated states in the cytoplasm. Following a receptor-mediated \([\text{Ca}^{2+}]_i\) increase, NFATs are dephosphorylated and activated by \(\text{Ca}^{2+}\)-dependent phosphatases, such as calcineurin [27]. Although still controversial, several studies have demonstrated that NFAT1 exhibits anti-apoptotic properties and promotes tumor progression [28, 29]. Our study indicated that gefitinib-mediated apoptosis and EGF-mediated NFAT1 induction are significantly decreased depending on \([\text{Ca}^{2+}]_e\) restriction in gefitinib-resistant cells. Thus, sustained NFAT1 induction in gefitinib-resistant cells might play a role in acquired drug resistance.

Our study demonstrated that NSCLC cells altered EGF-mediated \(\text{Ca}^{2+}\) signaling depending on gefitinib resistance. Importantly, restricting \([\text{Ca}^{2+}]_e\) in gefitinib-sensitive and gefitinib-resistant cells elicited identical \([\text{Ca}^{2+}]_i\) oscillations and significantly enhanced gefitinib sensitivity in gefitinib-resistant cells. Moreover, we showed the regulatory effects of \([\text{Ca}^{2+}]_e\) on NFAT1 induction and concluded that restricting \([\text{Ca}^{2+}]_e\) should be used to treat drug-resistant NSCLCs.

**Supporting information**

**S1 Fig. Expression of Orai1, STIM1, and SERCA2 in PC-9 and PC-9/GR cells.** PC-9 and PC-9/GR cells were respectively seeded on 60 mm culture dish and cultured in normal condition for overnight. Cells were lysed with RIPA buffer, and collected whole cell lysates were used for Western blot to determine the endogenous expression level of Orai1, STIM1, and SERCA2. Columns present the mean ± S.D. from 3 independent experiments. *\(P < 0.05\). (TIF)

**S1 Raw images.** (PPTX)

**Acknowledgments**

We would like to acknowledge Dr. Jin Kyung Rho at Korea university for providing the PC-9 and PC-9/GR cells for the study.

**Author Contributions**

**Conceptualization:** Mi Seong Kim, Sei Hoon Yang, Min Seuk Kim.

**Data curation:** Mi Seong Kim, So Hui Kim, Min Seuk Kim.

**Formal analysis:** Mi Seong Kim, Sei Hoon Yang, Min Seuk Kim.

**Funding acquisition:** Mi Seong Kim.

**Investigation:** Mi Seong Kim, So Hui Kim.

**Methodology:** So Hui Kim, Min Seuk Kim.

**Project administration:** Min Seuk Kim.

**Resources:** Sei Hoon Yang.

**Supervision:** Sei Hoon Yang, Min Seuk Kim.

**Validation:** Mi Seong Kim, So Hui Kim, Min Seuk Kim.

**Writing – original draft:** Min Seuk Kim.
References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA: a cancer journal for clinicians. 2017; 67 (1):7–30. Epub 2017/01/06. https://doi.org/10.3322/caac.21387 PMID: 28055103.

2. Rosell R, Bivona TG, Karachalios N. Genetics and biomarkers in personalisation of lung cancer treat- ment. Lancet. 2013; 382(9893):720–31. Epub 2013/08/27. https://doi.org/10.1016/S0140-6736(13) 61715-8 PMID: 23972815.

3. Pirker R. Novel drugs against non-small-cell lung cancer. Current opinion in oncology. 2014; 26(2):145– 51. Epub 2014/01/11. https://doi.org/10.1097/CCO.0000000000000056 PMID: 24406751.

4. Yamaoka T, Kusumoto S, Ando K, Ohba M, Ohmori T. Receptor Tyrosine Kinase-Targeted Cancer Therapy. International journal of molecular sciences. 2018; 19(11). Epub 2018/11/09. https://doi.org/10.3390/ijms19113491 PMID: 30404198; PubMed Central PMCID: PMC6274851.

5. Montor WR, Salas A, Melo FHM. Receptor tyrosine kinases and downstream pathway s as drugga ble targets for cancer treatment: the current arsenal of inhibitors. Molecular cancer. 2018; 17(1):55. Epub 2018/02/20. https://doi.org/10.1186/s12943-018-0792-2 PMID: 29455659; PubMed Central PMCID: PMC5817866.

6. Bryant JA, Finn RS, Slamon DJ, Cloughes y TF, Charles AC. EGF activates intracellular and intercellular calcium signaling by distinct pathways in tumor cells. Cancer biology & therapy. 2004; 3(12):1243 –9. Epub 2004/12/ 22. https://doi.org/10.4161/cbt.3.12.1233 PMID: 15611621.

7. Berridge MJ, Lipp P, Bootman MD. The versatility and universal ity of calcium signalli ng. Nature reviews Molecular cell biology. 2000; 1(1):11–21. Epub 2001/06/ 20. https://doi.org/10.1038/35036035 PMID: 11413485.

8. Sabourin J, Le Gal L, Saurwe in L, Haefliger JA, Raddatz E, Allagnat F. Store-operated Ca2+ Entry Mediated by Orai1 and TRPC1 Participates to Insulin Secretion in Rat beta-Cells. The Journal of biolog- ical chemist ry. 2015; 290(51):30 530–9. Epub 2015/10/24. https:// doi.org/10.1074/jbc.M115 .682583 PMID: 26494622; PubMed Central PMCID: PMC4683273.

9. Zhang P, Liu X, Li H, Chen Z, Yao X, Jin J, et al. TRPC5-induced autophagy promotes drug resistance in breast carcinoma via CaMKKbeta/AMPKalpha/mTOR pathway. Scientific reports. 2017; 7(1):3158. Epub 2017/06/11. https://doi.org/10.1038/s41598-017-03230-w PMID: 28600513; PubMed Central PMCID: PMC5466655.

10. Sun X, Wei Q, Cheng J, Bian Y, Tian C, Hu Y, et al. Enhanced Stim1 expression is associated with acquired chemo-resistance of cisplatin in osteosarcoma cells. Human cell. 2017; 30(3):216– 25. Epub 2017/03/ 23. https://doi.org/10.1007/s13577-017-0167-9 PMID: 28326487; PubMed Central PMCID: PMC5486860.

11. Kondratska K, Kondratskiy A, Yassine M, Lemonnier L, Lepage G, Morabito A, et al. Orai1 and STIM1 mediate SOCE and contribut e to apoptotic resistance of pancreatic adenocarcinoma. Biochimica et biophysica acta. 2014; 1843(10):2263–9. Epub 2014/03/04. https://doi.org/10.1016/j.bbamcr.2014.02.012 PMID: 24583265.

12. Mulder C, Prust N, van Doorn S, Reinecke M, Kuster B, van Bergen En Henegouwen P, et al. Adaptive Resistan ce to EGFR-Targeted Therapy by Calcium Signaling in NSCLC Cells. Molecular cancer research : MCR. 2018; 16(11):1773–84. Epub 2018/07/04. https://doi.org/10.1158/1541-7786.MCR-18 -0212 PMID: 29967110.

13. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na, II, Yang SH, et al. The role of MET activation in determin ing the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. Molecular cancer research: MCR. 2009; 7(10):1736 –43. Epub 2009/10/08. https://doi.org/10.1158/1541-7786.MCR-08-0504 PMID: 19808904.

14. Kim MS, Shin DM. Acidification induces OGR1/Ca(2+)/calpain signaling in gingival fibroblasts. Bio- chemical and biophysical research communications. 2018; 496(2):693–9. Epub 2018/01/26. doi: https://doi.org/10.1016/j.bbrc.2018.01.013 PMID: 29366789.

15. Kim MS, Muallem S, Kim SH, Kwon KB. Exosomal release through TRPML1-mediated lysosomal exo- cytosis is required for adipogenesis. Biochemical and biophysical research communications. 2019; 510 (3):409–15. Epub 2019/02/04. https://doi.org/10.1016/j.bbrc.2019.01.115 PMID: 3071251.

16. Wee P, Wang Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers. 2017; 9(5). Epub 2017/05/18. https://doi.org/10.3390/cancers9050052 PMID: 28513565; PubMed Centr al PMCID: PMC5447962.

17. Tiseo M, Bartolotti M, Gelsomino F, Bordi P. Emerging role of gefitinib in the treatment of non-small-cell lung cancer (NSCLC). Drug design, development and therapy. 2010; 4:81–98. Epub 2010/06/10. https://doi.org/10.2174/ddd.s6594 PMID: 20531963; PubMed Central PMCID: PMC2880339.

18. Chen ZL, Zhao SH, Wang Z, Qiu B, Li BZ, Zou F, et al. Expression and unique functions of four nuclear factor of activated T cells isoforms in non-small cell lung cancer. Chinese journal of cancer. 2011; 30
19. Stewart TA, Yapa KT, Monteith GR. Altered calcium signaling in cancer cells. Biochimica et biophysica acta. 2015; 1848(10 Pt B):2502–11. Epub 2014/08/26. https://doi.org/10.1016/j.bbamem.2014.08.016 PMID: 25150047.

20. Bergner A, Kellner J, Tufman A, Huber RM. Endoplasmic reticulum Ca2+-homeostasis is altered in Small and non-small Cell Lung Cancer cell lines. Journal of experimental & clinical cancer research: CR. 2009; 28:25. Epub 2009/02/25. https://doi.org/10.1186/1756-9966-28-25 PMID: 19236728; PubMed Central PMCID: PMC2653468.

21. Ay AS, Benzerdjeb N, Sevestre H, Ahidouch A, Ouaddi-Ahidouch H. Orai3 constitutes a native store-operated calcium entry that regulates non small cell lung adenocarcinoma cell proliferation. PloS one. 2013; 8(8):e72889. Epub 2013/09/24. https://doi.org/10.1371/journal.pone.0072889 PMID: 24058448; PubMed Central PMCID: PMC3772818.

22. Yang S, Zhang JJ, Huang XY. Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. Cancer cell. 2009; 15(2):124–34. Epub 2009/02/03. https://doi.org/10.1016/j.ccr.2008.12.019 PMID: 19185847.

23. Dupont G, Combettes L, Bird GS, Putney JW. Calcium oscillations. Cold Spring Harbor perspectives in biology. 2011; 3(3). Epub 2011/03/23. https://doi.org/10.1101/cshperspect.a004226 PMID: 21421924; PubMed Central PMCID: PMC3039928.

24. Zanotti S, Charles A. Extracellular calcium sensing by glial cells: low extracellular calcium induces intracellular calcium release and intercellular signaling. Journal of neurochemistry. 1997; 69(2):594–602. Epub 1997/08/01. https://doi.org/10.1046/j.1471-4159.1997.69020594.x PMID: 9231716.

25. Centeno PP, Herberger A, Mun HC, Tu C, Nemeth EF, Chang W, et al. Phosphate acts directly on the calcium-sensing receptor to stimulate parathyroid hormone secretion. Nature communications. 2019; 10(1):4693. Epub 2019/10/18. https://doi.org/10.1038/s41467-019-12399-9 PMID: 31619668; PubMed Central PMCID: PMC6795806.

26. Luo C, Shaw KT, Raghavan A, Aramburu J, Garcia-Cozar F, Perrino BA, et al. Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. Proceedings of the National Academy of Sciences of the United States of America. 1996; 93(17):8907–12. Epub 1996/08/20. https://doi.org/10.1073/pnas.93.17.8907 PMID: 8799126; PubMed Central PMCID: PMC38567.

27. Nguyen AH, Beland M, Gaitan Y, Bouchard M. Calcineurin a-binding protein, a novel modulator of the calcineurin-nuclear factor of activated T-cell signaling pathway, is overexpressed in wilms’ tumors and promotes cell migration. Molecular cancer research: MCR. 2009; 7(6):821–31. Epub 2009/06/18. https://doi.org/10.1158/1541-7766.MCR-08-0402 PMID: 19531566.

28. Zhang X, Zhang Z, Cheng J, Li M, Wang W, Xu W, et al. Transcription factor NFAT1 activates the mdm2 oncogene independent of p53. The Journal of biological chemistry. 2012; 287(36):30468–76. Epub 2012/07/13. https://doi.org/10.1074/jbc.M112.373738 PMID: 22787160; PubMed Central PMCID: PMC3436296.

29. Baumgart S, Giesel E, Singh G, Chen NM, Reutlinger K, Zhang J, et al. Restricted heterochromatin formation links NFATc2 repressor activity with growth promotion in pancreatic cancer. Gastroenterology. 2012; 142(2):388–98 e1-7. Epub 2011/11/15. https://doi.org/10.1053/j.gastro.2011.11.001 PMID: 22079596; PubMed Central PMCID: PMC3626431.