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Characterization of intracellular Ca^{2+} mobilization in gefitinib-resistant oral squamous carcinoma cells HSC-3 and -4

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Oral squamous cell carcinoma (OSCC) metastasis is characterized by distant metastasis and local recurrence. Combined chemotherapy with cisplatin and 5-fluorouracil is routinely used to treat patients with OSCC, and the combined use of gefitinib with cytotoxic drugs has been reported to enhance the sensitivity of cancer cells *in vitro*. However, the development of drug resistance because of prolonged chemotherapy is inevitable, leading to a poor prognosis. Therefore, understanding alterations in signaling pathways and gene expression is crucial for overcoming the development of drug resistance. However, the altered characterization of Ca^{2+} signaling in drug-resistant OSCC cells remains unclear. In this study, we investigated alterations in intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization upon the development of gefitinib resistance in human tongue squamous carcinoma cell line (HSC)-3 and HSC-4 using ratiometric analysis. This study demonstrated the presence of altered epidermal growth factor- and purinergic agonist-mediated $[Ca^{2+}]_i$ mobilization in gefitinib-resistant OSCC cells. Moreover, Ca^{2+} content in the endoplasmic reticulum, store-operated calcium entry, and lysosomal Ca^{2+} release through the transient receptor potential mucolipin 1, were confirmed to be significantly reduced upon the development of apoptosis resistance. Consistent with $[Ca^{2+}]_i$ mobilization, we identified modified expression levels of Ca^{2+} signaling-related genes in gefitinib-resistant cells. Taken together, we propose that the regulation of $[Ca^{2+}]_i$ mobilization and related gene expression can be a new strategy to overcome drug resistance in patients with cancer.

Keywords: Squamous cell carcinoma of head and neck, Gefitinib, Drug resistance, Calcium channel

Introduction

Gefitinib (ZD1839, Iressa™), a specific epidermal growth factor receptor (EGFR)–tyrosine kinase inhibitor, has been suggested to efficiently inhibit the proliferation of non–small cell lung cancer cells and head and neck squamous cell carcinoma (HNSCC) cells [1], and combinational use of gefitinib with cisplatin enhanced the apoptotic effect of cisplatin [2]. Moreover, a phase II study showed that monotherapy with gefitinib in 52 patients advanced–stage HNSCC patients showed effects equivalent to those of other chemotherapies [3]. Conventional chemotherapy using small–molecule anticancer drugs is implicated in improving the 1–year survival rates; however, this is substantially limited by the development of drug resistance. Accumulating evidence indicates that the development of acquired drug resistance is accompanied by differential gene

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expression, which sequentially mediates altered signaling pathways [4,5]. Microarray analysis revealed that prolonged exposure of cancer cells to anti-cancer drugs led to differential gene expression profiles, which are associated with DNA repair, mitogen-activated protein kinase pathway, epithelial to mesenchymal transition, and intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling [4-6].

Altered $[Ca^{2+}]_i$ mobilization and dysregulation of Ca^{2+} homeostasis in cancer cells are well documented to mediate diverse cancer cell responses, including angiogenesis, autophagy, and invasion [7]. In pathophysiological conditions, such as tumorigenesis, altered Ca^{2+} signaling is frequently observed, which leads to excessive proliferation and apoptosis resistance. Up-regulation of stromal interaction molecule 1 (STIM1) and ORAI1 in 5-fluorouracil (5-FU)- and gemcitabine-treated pancreatic adenocarcinoma cells resulted in enhanced store-operated Ca^{2+} influx, which enhanced apoptotic resistance [8]. Over-expression of plasma membrane calcium ATPase 2 (PMCA2), which pumps $[Ca^{2+}]_i$ to outside the cells, attenuates apoptotic cell death in breast cancer cells [9]. Cav3.1, a subfamily of T-type Ca^{2+} channels, is highly expressed in oral squamous cell carcinoma (OSCC) and is associated with anti-apoptotic activity [10]. This is in line with evidence suggesting that alterations in Ca^{2+} signaling are closely involved in the development of anti-cancer drug resistance in diverse cancer cells; however, altered Ca^{2+} signaling in apoptosis-resistant OSCC cells has not been well characterized.

In the present study, we established gefitinib-resistant cell lines using OSCC cells, such as human tongue squamous carcinoma cell line (HSC)-3 and HSC-4 cells, and altered $[Ca^{2+}]_i$ mobilization and Ca^{2+} signaling-related gene expression upon the development of gefitinib resistance. The identification of alterations in Ca^{2+} signaling in apoptosis-resistant cells may provide new strategies to overcome drug resistance in cancers.

Materials and Methods

1. Cell culture and reagents

The human OSCC cell lines HSC-3 and HSC-4, which were provided by Dr. Sung-Dae Cho, Seoul National University, were used in the present study (06/16/2020). Each cell line was cultured in DMEM/F12 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Amarillo, TX, USA) at 37℃ in 5% CO_2. Mycoplasma screening was performed every 4 months after thawing using the end-point polymerase chain reaction (PCR) method (BioMycoX Mycoplasma PCR Detection Kit; Cellsafe, Yongin, Korea).

Gefitinib and antibodies against Stim1, sarco/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2), and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against EGFR, Orai1, and PMCA were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Human epidermal growth factor (EGF), adenosine triphosphate (ATP), ML-SA1, and antibody against transient receptor potential mucolipin 1 (TRPML1) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cyclopiazonic acid (CPA) was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2. Establishment of gefitinib-resistant cells

Gefitinib-resistant HSC-3 and HSC-4 cell lines (HSC-3/GR and HSC-4/GR) were established by repetitive cycles of exposure to sequential concentrations of gefitinib (0.01, 50, 20, and 10 μM). Following treatment with each concentration of gefitinib, the cells were restored for 18 days in the absence of gefitinib. After sequential exposure to gefitinib, surviving cells were maintained in culture medium containing 1 μM gefitinib for 4 months. To determine apoptosis resistance, a cell proliferation assay was conducted at each subculture.

3. Western blot assay

Protein expression levels were determined by western blot analysis following a standard protocol. Briefly, cells were lysed with RIPA buffer (Invitrogen, Foster City, CA, USA) containing proteases and a phosphatase inhibitor cocktail. Whole cell lysates were separated by SDS-PAGE and separated by molecular weight using gel electrophoresis. After transfer onto PVDF membranes (0.2 μm pore size), the membrane was incubated with the primary antibodies, EGFR (1:1,000), Orai1 (1:1,000), Stim1 (1:1,000), SERCA2 (1:1,000), TRPML1 (1:1,000), PMCA (1:1,000), and β-actin (1:1,000), and HRP-conjugated IgG was used as the secondary antibody. Immunoreactive proteins were detected using AzureSpot 2.0 (Azure Biosystems, Inc., Dublin, CA, USA).

4. Cell proliferation assay

Cell proliferation was assessed using EZ-CYTOX (Daeil Lab Service Co. Ltd., Seoul, Korea) according to the manufacturer's

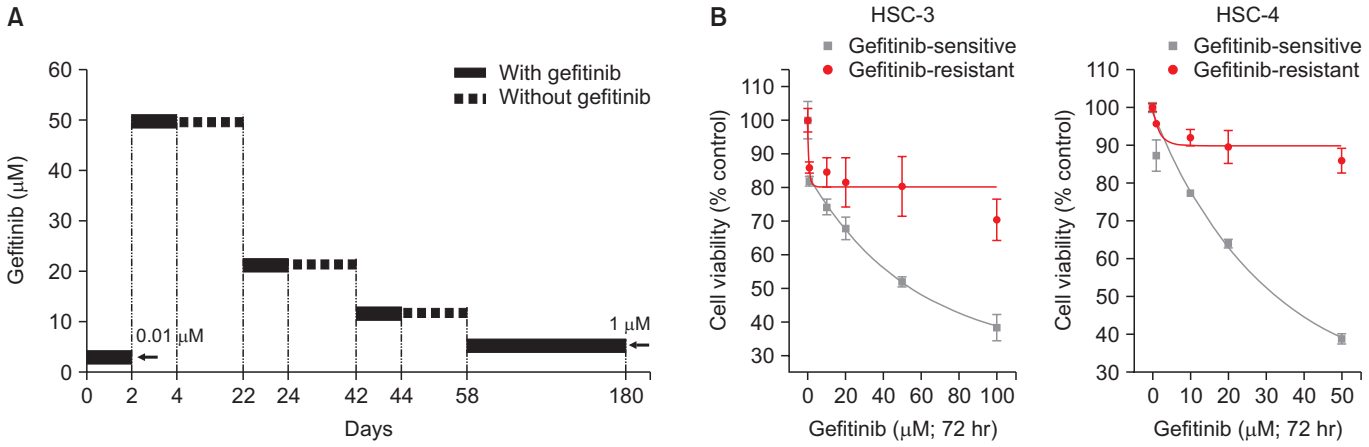

Fig. 1. Establishment of gefitinib-resistant cell lines, and comparison of cell viability of each cell line responding to gefitinib. (A) Flow chart of the protocol for establishing gefitinib-resistant cells. HSC-3 and HSC-4 cells were incubated for the indicated period in the presence of gefitinib (0.01, 50, 20, 10, and 1 μM). Following each step of incubation, cells were applied to cell viability assay. (B) Each cell line, including HSC-3 and HSC-4 and gefitinib-resistant sub-cell lines, HSC-3/GR and HSC-4/GR, was treated with gefitinib for 72 hours in a dose-dependent manner, and cell viability was measured. Data are presented as % control (HSC-3 and HSC-4).
HSC, human tongue squamous carcinoma cell line.

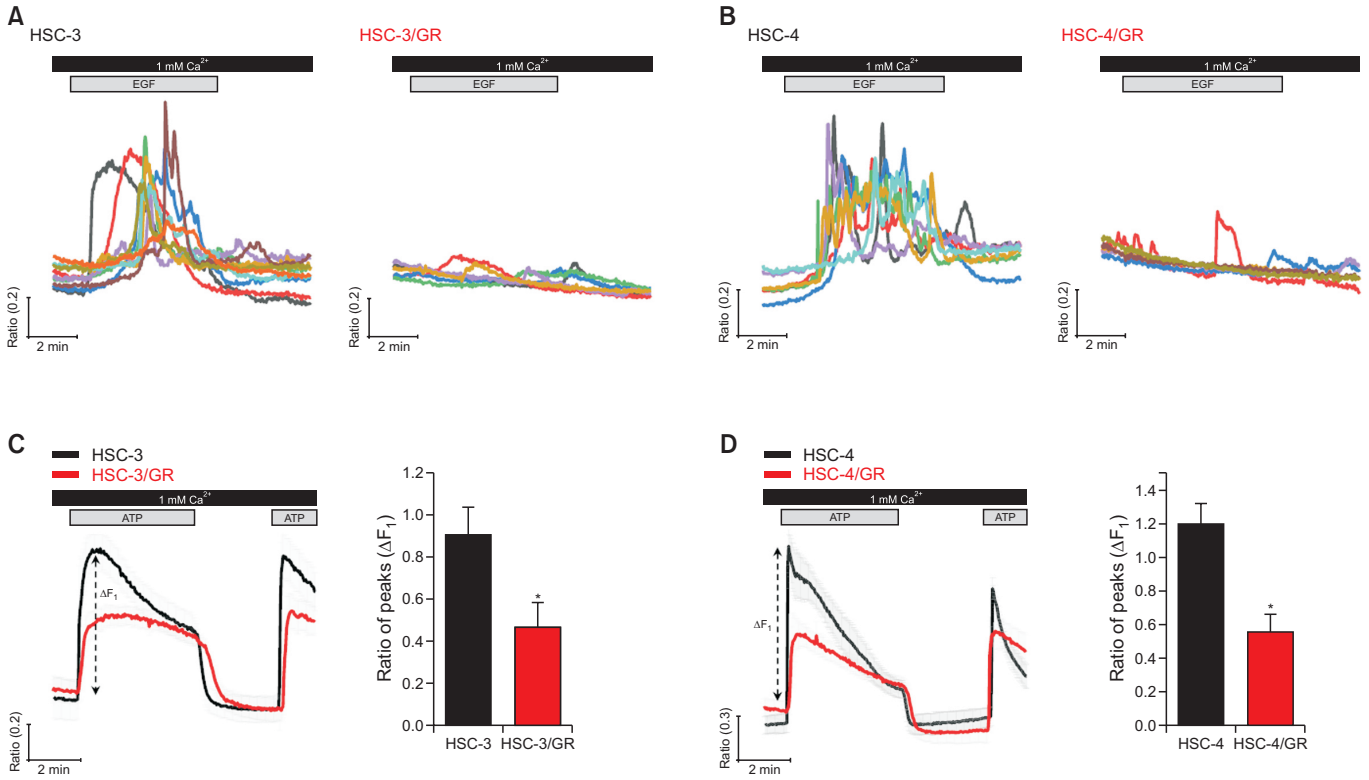

Fig. 2. Characterization of EGF- and ATP-mediated $[Ca^{2+}]_i$ mobilization in gefitinib-sensitive and -resistant cells. Cells were loaded with Fura-2, a fluorescent dye for indicating free Ca^{2+} ions, for 1 hour and applied to monitor intracellular $[Ca^{2+}]_i$ mobilization in a single cell. EGF and ATP diluted in regular HEPES buffer were perfused to the cells for the indicated period. Each line represents $[Ca^{2+}]_i$ mobilization in a single cell, and data are shown as value of ratio (F_{340}/F_{380}). (A) EGF-mediated $[Ca^{2+}]_i$ mobilization in HSC-3 and HSC-3/GR cells and (B) HSC-4 and HSC-4/GR cells were monitored. (C) ATP-mediated $[Ca^{2+}]_i$ mobilization in HSC-3 and HSC-3/GR and (D) HSC-4 and HSC-4/GR cells were measured. Transient Ca^{2+} increase by ATP (ΔF_1) is presented as mean ± standard deviation. n = 10.
EGF, epidermal growth factor; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+}; HSC, human tongue squamous carcinoma cell line.
*$p < 0.05$.

instructions. Briefly, cells were plated in a 96-well plate and treated under the indicated conditions. Following the additional incubation, EZ-CYTOX (10 μL) was added to each well, and the cells were incubated for 30 minutes. Optical density was measured at 450 nm using an iMAX Microplate Reader (Bio-Rad, Hercules, CA, USA).

5. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using a Ca^{2+}-sensitive fluorescence dye, Fura-2/AM (Sigma-Aldrich). Cells loaded with Fura-2 (5 μM) were perfused with regular HEPES buffer containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose (adjusted to pH 7.4, 310 mOsm). Next, 1 mmol/L $CaCl_2$ was replaced with 1 mmol/L EGTA in Ca^{2+}-free HEPES buffer. After a brief wash out with regular HEPES buffer, Fura-2 fluorescence was monitored using 340 and 380 nm dual-wavelength excitation, and the emission wavelength at 510 nm was measured using a sCMOS camera (Andor Technology Ltd., Belfast, UK). The collected data were analyzed using the MetaFluor Fluorescence Ratio Imaging software (Molecular Devices, San Jose, CA, USA) and presented as the F_{340}/F_{380} ratio.

6. Statistical analysis

Statistical analysis was conducted using the Origin software (OriginLab Corporation, Northampton, 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 analysis of variance followed by Tukey's post-hoc test and t-test. Statistical significance was set at $p < 0.05$.

Results

1. Establishment of gefitinib-resistant sub-cell line of HSC-3 and HSC-4

To investigate alterations in $[Ca^{2+}]_i$ mobilization in anti-cancer drug-resistant OSCC cells, we first established gefitinib-insensitive HSC-3 and HSC-4 cell lines. Gefitinib-resistant cell lines were established by repetitive cycles of exposure to gefitinib from the sensitive parental cells (HSC-3 and HSC-4) over a 6-month period (Fig. 1A). Each cell line was used for the cell viability assay to determine the development of gefitinib-resistance. HSC-3 and HSC-4 cells were exposed to gefitinib (1, 10, 20, 50, and 100 μM) for 72 hours. Fig. 1B shows that

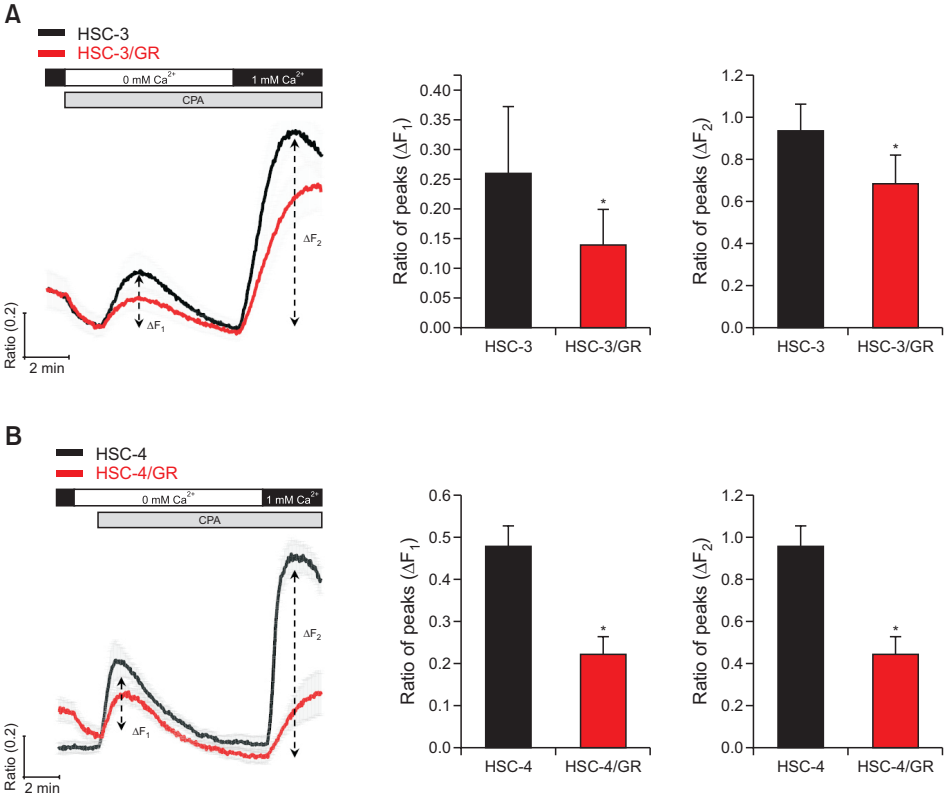

Fig. 3. Reduction of ER Ca^{2+} content and SOCE in gefitinib-resistant cell lines. Each cell line and gefitinib-resistant sub-cell line were loaded with Fura-2 and applied to measure ER Ca^{2+} content (ΔF_1) and SOCE (ΔF_2) in response to cyclopiazonic acid (CPA) and extracellular Ca^{2+} restoration, respectively. (A) HSC-3 and HSC-3/GR cells and (B) HSC-4 and HSC-4/GR cells were sequentially treated with CPA in the absence and presence of extracellular Ca^{2+}. ER Ca^{2+} content (ΔF_1) and SOCE (ΔF_2) are shown as mean ± standard deviation. n = 10.
ER, endoplasmic reticulum; SOCE, store-operated Ca^{2+} entry; HSC, human tongue squamous carcinoma cell line.
*$p < 0.05$.

the cell viability of both HSC-3 and HSC-4 cells is significantly decreased in response to gefitinib in a dose-dependent manner. In contrast, gefitinib-resistant sub-cell lines, hereafter referred to as HSC-3/GR and HSC-4/GR, were shown to be insensitive to gefitinib.

2. EGF- and ATP-mediated $[Ca^{2+}]_i$ mobilization is decreased in gefitinib-resistant cells

EGFR is reported to be overexpressed in the majority of OSCC patients [11], and activation of EGFR transduces diverse downstream signaling pathways, including $[Ca^{2+}]_i$ mobilization. In the present study, we first examined the characteristics of EGF-mediated $[Ca^{2+}]_i$ mobilization in gefitinib-sensitive and-resistant OSCC cells. Cells loaded with Fura-2 were perfused with 200 ng/mL EGF diluted in HEPES buffer for the indicated time. Figs. 2A and 2B show that treatment of each cell line with EGF (200 ng/mL) only elicits transient $[Ca^{2+}]_i$ mobilization in gefitinib-sensitive cells, but not in gefitinib-resistant cells, HSC-3/GR and HSC-4/GR. The right panels of Figs. 2A and 2B indicate that EGF-mediated $[Ca^{2+}]_i$ responses are considerably limited in HSC-3/GR and HSC-4/GR cells. Furthermore, $[Ca^{2+}]_i$ mobilization (ΔF_1) by stimulation with the purinergic agonist ATP was significantly reduced in both gefitinib-resistant cells compared to that in gefitinib-sensitive cells (0.9102 ± 0.12644 vs. 0.46931± 0.11426 for HSC-3 and HSC-3/GR, 1.20416 ± 0.11248 vs. 0.5642 ± 0.09652 for HSC-4 and HSC-4/GR; Figs. 2C and 2D).

3. Endoplasmic reticulum Ca^{2+} content and store-operated Ca^{2+} entry is reduced in gefitinib-resistant cells

Store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} influx in non-excitable cells that mediates diverse cellular responses [12]. To deplete the endoplasmic reticulum (ER) Ca^{2+} content and activate SOCE, each cell line was sequentially treated with 25 μM CPA, an inhibitor of SERCA, diluted in Ca^{2+}-free HEPES buffer, and 25 μM CPA diluted in regular HEPES buffer containing 1 mM $CaCl_2$. Fig. 3 show that the levels of ER Ca^{2+} (ΔF_1) and SOCE (ΔF_2) are significantly decreased in HSC-3/GR and HSC-4/GR cells compared to those in gefitinib-sensitive cells (ΔF_1: 0.26015 ± 0.11179 vs. 0.14043 ± 0.05948 and ΔF_2: 0.93727 ± 0.12236 vs. 0.68543 ± 0.13577 for HSC-3 and HSC-3/GR, ΔF_1: 0.46016 ± 0.06875 vs. 0.22768 ± 0.04635 and ΔF_2: 0.94713 ± 0.08599 vs. 0.32349 ± 0.11882 for HSC-4 and HSC-4/GR).

4. Lysosomal Ca^{2+} release through TRPML1 is decreased in gefitinib-resistant cells

Next, we evaluated the level of lysosomal Ca^{2+} release into the cytosol through TRPML1 between gefitinib-sensitive and-resistant cell lines. We applied 20 μM ML-SA1 diluted in regular HEPES buffer to each cell line, and ML-SA1-mediated $[Ca^{2+}]_i$ elevation was then measured. Fig. 4 show that ML-SA1-mediated $[Ca^{2+}]_i$ (ΔF_1) in HSC-3 and HSC-4 cells (0.65744 ± 0.26259 and 0.26389 ± 0.07571) is reduced to 0.16852 ± 0.06306 and 0.14147 ± 0.079, respectively.

5. Expression level alterations of Ca^{2+} signaling-related genes

Considering the previous results, we compared the expres-

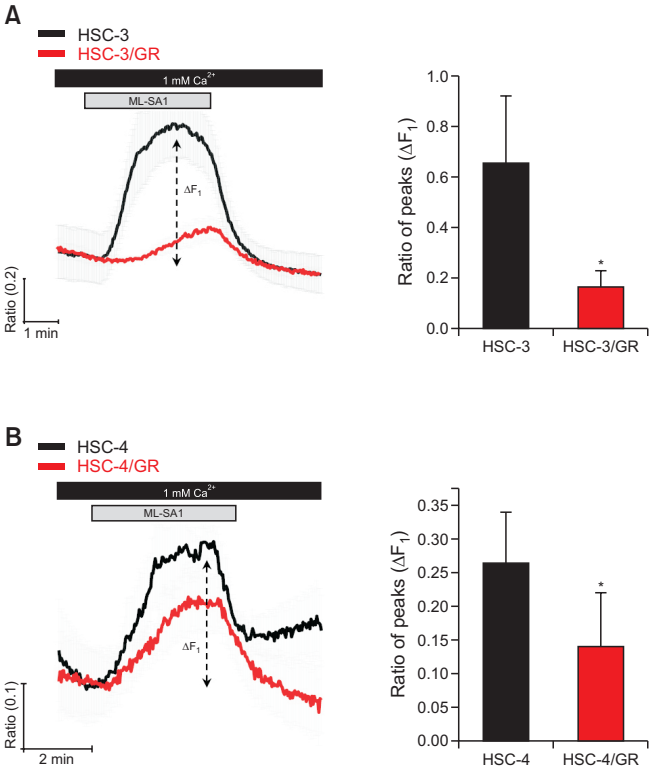

Fig. 4. Reduction of lysosomal Ca^{2+} release through TRPML in gefitinib-resistant cell lines. Each cell line and gefitinib-resistant sub-cell line were loaded with Fura-2 and applied to measure lysosomal Ca^{2+} release through TRPML (ΔF_1) in response to ML-SA1. (A) HSC-3 and HSC-3/GR cells and (B) HSC-4 and HSC-4/GR cells were treated with ML-SA1 in the presence of extracellular Ca^{2+}. Lysosomal Ca^{2+} release (ΔF_1) is shown as mean ± standard deviation. n = 10.
TRPML, ransient receptor potential mucolipin; HSC, human tongue squamous carcinoma cell line.
*$p < 0.05$.

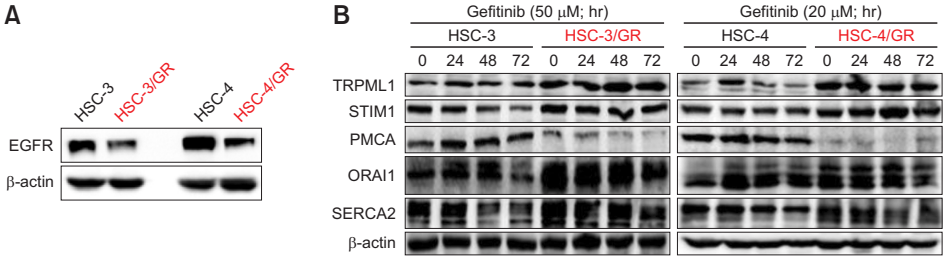

Fig. 5. Alterations of Ca^{2+} signaling-related gene expression in gefitinib-resistant cells. (A) Whole cell lysates of HSC-3 and HSC-3/GR and HSC-4 and HSC-4/GR were used for determining the EGFR expression level. (B) Each cell line, including gefitinib-resistant cell lines, was treated with gefitinib for the indicated time. Following the incubation, whole cell lysates were collected, and applied to detect the expression levels of TRPML1, Stim1, PMCA, Orai1, and SERCA2. β-actin was used as a loading control for each sample.
HSC, human tongue squamous carcinoma cell line; EGFR, epidermal growth factor receptor; TRPML1, transient receptor potential mucolipin 1; PMCA, plasma membrane calcium ATPase; SERCA2, sarco/endoplasmic reticulum Ca^{2+} ATPase 2.

sion levels of Ca^{2+} signaling-related genes, such as EGFR, STIM1, ORAI1, TRPML1, PMCA, and SERCA2, between gefitinib-sensitive and-resistant cells. Each cell line was incubated under the indicated conditions, and whole-cell lysates were used to determine gene expression. As shown in Fig. 5, we found that endogenous levels of EGFR and PMCA in HSC-3/GR and HSC-4/GR cells are lower than those in HSC-3 and HSC-4 cells, respectively. In contrast, expression levels of TRPML1, STIM1, ORAI1, and SERCA2 were higher in gefitinib-resistant cells than in gefitinib-sensitive cells (Fig. 5B). Notably, the expression levels of STIM1 and SERCA2 appeared to be reduced in response to gefitinib treatment (Fig. 5B).

Discussion

OSCC is a prevalent cancer worldwide and is characterized by distant metastasis and local recurrence. Metastasis to regional lymph nodes is frequently observed in advanced stages of OSCC, which is related to the poor prognosis of OSCC patients [13]. Combined treatment with anti-cancer drugs, such as cisplatin and 5-FU, is routinely used clinically in OSCC cases, and the combined use of gefitinib with cytotoxic drugs enhances the sensitivity of cancer cells *in vitro* [14]. However, the development of apoptosis resistance by prolonged treatment with anti-cancer drugs is inevitable. Well-documented evidence indicates that alterations in $[Ca^{2+}]_i$ signaling are associated with the development of drug resistance in various cancer cells [4], however, the characteristics of altered $[Ca^{2+}]_i$ mobilization in drug-resistant OSCC cells remain unclear. The present study discloses the altered $[Ca^{2+}]_i$ mobilization and Ca^{2+} signaling-related gene expression in gefitinib-resistant cells using HSC-3 and -4 cell lines. First, EGF-mediated $[Ca^{2+}]_i$ mobilization was attenuated in gefitinib-resistant HSC-3 and -4 cells (Figs. 2A and 2B). EGFR overexpression and amplification of EGFR-mediated signaling in OSCC and lung cancer have been frequently reported [15]. Moreover, EGF-mediated $[Ca^{2+}]_i$ oscillations are a major factor in activating proliferation-, migration-, and invasion-related signaling in cancer cells [16]. Our results show that EGF-mediated $[Ca^{2+}]_i$ mobilization is attenuated by the development of gefitinib resistance in HSC-3 and -4 cells, suggesting that the EGFR expression level and downstream pathways of EGFR might be modified. Consistent with this, we also found that purinergic stimulation with ATP in OSCC cells resulted in altered $[Ca^{2+}]_i$ mobilization upon the development of gefitinib resistance (Figs. 2C and 2D). Accumulating evidence has demonstrated that high concentrations of purinergic agonists, including ATP, are a hallmark of the tumor microenvironment, and activation of purinergic receptors subsequently transduces diverse signals leading to the regulation of inflammasomes, cell proliferation, and migration in cancer cells [17-19]. Considering our results, the development of gefitinib resistance in HSC-3 and -4 cells appears to reduce the signaling pathways underlying purinergic activation.

In non-excitable cells, extracellular Ca^{2+} influx through store-operated Ca^{2+} channels, including Orai1, is a major Ca^{2+} source that mediates diverse Ca^{2+}-mediated signaling pathways [20]. Identifying the alterations of EGF- and ATP-mediated $[Ca^{2+}]_i$ mobilization led us to characterize SOCE in gefitinib-sensitive and-resistant OSCC cells. The present study showed that both ER Ca^{2+} content and SOCE were significantly decreased in gefitinib-resistant cells compared to those in gefitinib-sensitive cells (Fig. 3). Interestingly, numerous studies have reported the elevation of SOCE and related gene expression in tumorigenesis, whereas downregulation of Orai1 and SOCE in apoptosis-resistant cancer cells was frequently observed [5,21]. Kondratska et al. [8] reported that knockdown of Orai1

and Stim1, major components of SOCE, enhanced anti-cancer drug-mediated apoptosis in pancreatic adenocarcinoma. Consistent with these reports, our results prove that reduced ER Ca^{2+} content and SOCE are associated with the development of gefitinib resistance in OSCC cells.

Other intracellular organelles, including endosomes and lysosomes, have recently been regarded as $[Ca^{2+}]_i$ stores [22]. In particular, lysosomal Ca^{2+}- and Ca^{2+}-mediated lysosomal exocytosis play key roles in cancer progression [23]. TRPML1, which is localized in lysosomes and responsible for Ca^{2+}-mediated lysosomal exocytosis, is overexpressed in cancer, particularly in HNSCC [24]. Based on these previous reports, we compared the lysosomal Ca^{2+} release through TRPMLs in gefitinib-sensitive and gefitinib-resistant OSCC cells, and confirmed that lysosomal Ca^{2+} release through TRPMLs is significantly decreased in gefitinib-resistant cells (Fig. 4). Taken together, our data suggest that lysosomal Ca^{2+}-mediated cellular responses are attenuated in gefitinib-resistant cells.

Lastly, we investigated whether expression levels of Ca^{2+} signaling-related genes, such as EGFR, TRPML1, Stim1, PMCA, Orai1, and SERCA2, are modified in gefitinib-resistant cells in comparison to those in gefitinib-sensitive cells. The present study shows that the levels of EGFR and PMCA are reduced by the development of gefitinib resistance. In contrast, the expression levels of TRPML1, Stim1, Orai1, and SERCA2 were surprisingly elevated in gefitinib-resistant cells. Considering the $[Ca^{2+}]_i$ mobilization results, we expected a reduction in TRPML1, Orai1, and Stim1 in gefitinib-resistant cells. Although we did not study this in detail, we assume that expressed proteins in gefitinib-resistant cells may accumulate in the ER or endosomes during endocytosis. However, further studies are required to confirm this hypothesis.

In summary, the present study provides evidence of altered EGF- and purinergic agonist-mediated $[Ca^{2+}]_i$ mobilization in gefitinib-resistant OSCC cells. Moreover, ER Ca^{2+} content, SOCE, and lysosomal Ca^{2+} release through TRPMLs were confirmed to be significantly reduced upon the development of apoptosis resistance. Consistent with $[Ca^{2+}]_i$ mobilization, we identified the modified expression levels of Ca^{2+} signaling-related genes, including EGFR, in gefitinib-resistant cells. Taken together, we propose that regulation of $[Ca^{2+}]_i$ mobilization and related gene expression can be a new strategy to overcome drug resistance in cancers.

Acknowledgements

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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