Abstract. The development of cutaneous squamous cell carcinoma (cSCC) is associated with activation of the epidermal growth factor receptor (EGFR). EGFR-targeting presents a promising strategy for improving therapeutic efficacy. However, recent studies have suggested that tumours overexpressing EGFR depend on autophagy for survival and exhibit resistance to EGFR-targeting drugs. Chloroquine diphosphate (CQ), an autophagy inhibitor that may enhance the cytocidal effect of gefitinib against cSCC, was used in the present study. Cytotoxicity assays were performed to determine the half-maximal inhibitory concentration values of gefitinib and CQ in A431 cells. Drug interaction was analysed using CompuSyn software, which also determined combination index and dose reduction index values. Apoptosis and autophagy of A431 cells were investigated via flow cytometry, western blotting analyses, acridine orange/ethidium bromide staining and monodansylcadaverine staining. Suppression of autophagy by CQ, which was demonstrated by an alteration in microtubule associated protein 1 light chain 3-B in CQ pre-treated A431 cells, significantly enhanced cell apoptosis, which suggested that gefitinib-induced autophagy is cytoprotective. Thus, CQ was demonstrated to exhibit a synergistic apoptotic effect when used in combination with gefitinib during cSCC therapy. Further in vivo investigations are required to confirm the results of the present study.

Introduction

Cutaneous squamous cell carcinoma (cSCC), also known as squamous-cell skin cancer, is a malignancy originating from keratinocytes in the epidermis or epidermal appendages (1). cSCC represents the second most common type of non-melanoma skin cancer following basal cell carcinoma and accounts for ~20% of all cutaneous malignancies (2). Exposure to chronic ultraviolet radiation is considered to be a risk factor for the development of cSCC, which is associated with a notable alteration in EGFR expression (3,4). EGFR, a receptor tyrosine kinase, serves an important regulatory role in the Ras/mitogen-activated protein kinase, phosphoinositide 3-kinase/protein kinase B and phospholipase C pathways in squamous cells (5), which are involved in cell apoptosis, proliferation, invasion, metastasis and angiogenesis (6). However, the deregulation of EGFR activation has been associated with the development and progression of cSCC (7). Thus, an increasing number of studies have investigated EGFR-targeted therapies in recent years, including monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs) (8-10). mAbs, which include cetuximab, panitumumab, nimotuzumab and zalutumab, block the extracellular downstream signalling pathway (11). In the present study, the cytotoxic and apoptotic effects exhibited by A431 cells treated with gefitinib were investigated.

Cells and tumours overexpressing EGFR have been demonstrated to exhibit dysregulated autophagy (12), resulting in cells degrading and recycling cellular constituents (13). The exact role of autophagy is unknown. It has been suggested that autophagy represents an alternative tumour-suppressing mechanism and is associated with...
genomic instability, suppression of cell growth and degradation of important cellular components (14). Recycled proteins and energy contribute to the maintenance of cellular homeostasis and increase the survival of tumor cells under stress conditions (15). However, it has been reported that autophagy represents a survival strategy exhibited by skin cancer cells in response to cisplatin, an adjuvant chemotherapy used for the treatment of patients with invasive cSCC (16). Recently, numerous studies have demonstrated that autophagy represents an important mechanism associated with resistance to TKIs (17,18). It has also been revealed that inhibition of autophagy enhances the anti-cancer effect of EGFR inhibitors in human bladder cancer cells (19). Furthermore, targeting autophagy in triple negative breast cancer cells is an effective treatment for the enhancement of sensitivity to EGFR inhibitors (20). However, to the best of our knowledge, the role of autophagy associated with the administration of gefitinib as a neoadjuvant treatment followed by surgery and/or radiotherapy for the treatment of patients with aggressive cSCC has not been clearly determined.

To determine the effects of autophagy on the cytoprotection of gefitinib-treated A431 cells, chloroquine diphosphate (CQ), an inhibitor of autophagosome formation was used in the present study to inhibit autophagy. The results demonstrated that gefitinib induced caspase-dependent apoptosis and activated the autophagic response in A431 cells. In addition, the role of autophagy in sSCC cell survival, was examined by assessing the anti-proliferative effect following co-treatment with CQ and gefitinib.

Materials and methods

Cell culture. The cSCC cell line A431 (derived from an 85-year-old female patient suffering from vulvar squamous cell carcinoma; China Centre for Type Culture Collection and Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, 100 units/ml penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37˚C in a humidified atmosphere.

Reagents and antibodies. Gefitinib (cat. no. S1025) was purchased from Selleck Chemicals (Houston, TX, USA) and CQ (cat. no. A506569) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Gefitinib and CQ were dissolved in DMSO and DMEM, respectively, and subsequently stored at a stock concentration of 100 mM at -20˚C. The following reagents were purchased from Selleck Chemicals (Houston, TX, USA) and Invitrogen; Thermo Fisher Scientific, Inc.; 1:5,000) and HRP-tagged anti-rabbit IgG (cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.; 1:5,000).

Cytotoxicity assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to perform cytotoxicity assays. Cells were plated in triplicate in 96-well plates at a density of 8x10³ cells/well and cultured overnight. Media was then removed via suction with an aspirator and replaced with 0.1 ml fresh DMEM containing different concentrations of gefitinib (0, 10, 20, 30, 40 or 50 µM) or CQ (0, 50, 100, 150, 200, 250 or 300 µM). Control cells were treated with the same volumes of DMSO or DMEM as the experimental groups. Following this, the plates were incubated at 37˚C for 12 h. Each well was subsequently incubated with 100 µl DMEM medium containing 10 µl CCK-8 for 2 h. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and half-maximal inhibitory concentration (IC₅₀) values were calculated based on log values using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Drug combination analysis. Gefitinib and CQ were added separately and together in a constant ratio, as calculated from a dose-effect curve. Inhibition effect was scored from 0 to 1, where a score of 0 represented no effect and a score of 1 represented 100% effect. CompuSyn software (version 1.0; T. C. Chou and N. Martin, Memorial Sloan-Kettering Cancer Centre, New York) was used to calculate the combination index (CI) and an isobologram was established to quantitatively determine the effect of drug interactions.

Investigation of apoptotic morphology via fluorescent microscopy. Following the treatment of A431 cells with either gefitinib (20 µM), CQ (188 µM) or gefitinib (20 µM) + CQ (188 µM) at 37˚C for 12 h, morphological observations of apoptosis and cell death were investigated using acridine orange/ethidium bromide staining (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Following incubation, cells were washed with PBS and subsequently fixed with 4% formaldehyde at room temperature for 15 min. Fixed cells were again washed with PBS and stained with acridine orange/ethidium bromide at room temperature for 5 min. Stained cells were subsequently observed and imaged under a Ti-Eclipse inverted fluorescent microscope (Nikon Corporation, Tokyo, Japan; magnification, x10).

Annexin V/propidium iodide (PI) staining assay for apoptosis. Following treatment with either gefitinib (20 µM), CQ (188 µM) or gefitinib (20 µM) + CQ (188 µM) at 37˚C for 12 h, A431 cells were collected and washed three times using ice-cold PBS. Cells were then resuspended in 400 µl binding buffer and subsequently incubated with 5 µl Annexin V-FITC and 5 µl PI at room temperature for 15 min in the dark. Following this, flow cytometric analysis was immediately performed and data was analysed using CellQuest software (version 5.1; BD Biosciences, San Jose, CA, USA).

Monodansylcadaverine (MDC) staining for the identification of autophagic vacuoles. Autophagic vacuoles were stained as previously described (21,22). A431 cells were seeded in
24 well-plate at a density of 3x10^4 cells/well. Following a 12 h incubation with gefitinib (20 µM) and CQ (188 µM), either alone or in combination at 37°C, cells were cultured in 50 µM MDC for 15 min at 37°C. Cells were then washed with PBS (pH 7.4), and levels of fluorescence were subsequently measured and imaged using an inverted fluorescence microscope (Nikon Eclipse Ti; Nikon Corporation; magnification, x20). All experiments were repeated at least three times.

Western blotting. Cells were treated with CQ (188 µM) and/or Ge (0–40 µM) for 0–12 h at 37°C. Plates were subsequently washed twice with ice-cold PBS and the cells were lysed using radioimmunoprecipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were quantified using Bradford reagent. Denatured proteins (20 µg/well) were separated by 12% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following this, membranes were blocked at room temperature for 1 h in blocking buffer containing 5% dried skimmed milk that was diluted with TBST containing 0.1% Tween 20. Membranes were then incubated with primary antibodies against LC3-II, PARP, caspase-3, and α-tubulin at 4°C overnight. Following this, membranes were washed with TBST and then incubated with HRP-conjugated secondary antibodies (1:5,000 in 0.1% TBST) for 90 min at room temperature. Membranes were again washed with TBST and immune complexes were then detected using enhanced chemiluminescence reagents (cat. no. WBLUF0500; Merck KGaA, Darmstadt, Germany). Densitometry of the western blot bands was performed using ImageJ software (v1.52i; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Differences between groups were compared using two-tailed Student's t tests or one-way analysis of variance followed by Student-Newman-Keuls-q post hoc test. Data were analysed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and presented as means ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

Gefitinib and CQ induce cytotoxic effects in A431 cells. To investigate the cytotoxicity of gefitinib and CQ, A431 cells were treated with various doses of gefitinib or CQ for 12 h. The results revealed that gefitinib and CQ both induce cytotoxic effects in A431 cells in a dose-dependent manner (Fig. 1A and B). Following 12 h of treatment, the IC_{50} values of gefitinib and CQ in A431 cells were demonstrated to be 19.77±1.76 and 189.1±3.29 Mm, respectively.

Gefitinib and CQ synergistically inhibit the proliferation of A431 cells. Combinatory administration of gefitinib and CQ was investigated using CompuSyn software. Concentrations of 10, 15, 20, 30 and 40 µM, and 94, 141, 188, 282 and 376 µM were used to establish a dose-effect curve for gefitinib and CQ, respectively. A constant ratio (20/188=5:47) was used to establish the doses used in combinatorial treatment groups (10+94, 15+141, 20+188, 30+282 and 40+376 Mm). Gefitinib and CQ exhibited a synergistic effect (Fig. 2A-E). The dose-response effects of gefitinib, CQ and gefitinib + CQ are presented in Fig. 2A. CI values, a quantitative definition for synergism, were revealed to be <1 in A431 cells, which indicated that combinatorial treatment with gefitinib and CQ exhibited synergistic cytotoxic effects in A431 cells (Fig. 2B). Isobolograms, representing equipotent combinations of two drugs administered at different dosages, were also established via CompuSyn analysis. The dosages of drug combinations revealed by the isobologram also suggested that gefitinib and CQ exhibited synergistic effects in A431 cells (Fig. 2C). Dose reduction index (DRI) values of each drug in combination treatment, which measures the number of folds by which single drug doses can be reduced by when used in combination, were revealed to be >1, thus indicating a favourable drug combination (Fig. 2D). Data obtained via CompuSyn analyses are presented in Fig. 2E.

Gefitinib and CQ induce apoptosis via the caspase-dependent apoptosis pathway. To determine the mechanism of cell death induced in A431 cells following combinatory treatment with gefitinib and CQ, acridine orange/ethidium bromide staining assays were performed. The results demonstrated that the number of apoptotic cells (early apoptotic cells with yellow-green fluorescence and late apoptotic cells with orange fluorescence) in gefitinib + CQ treatment groups were markedly increased compared with cells treated with gefitinib or CQ alone (Fig. 3A). To determine the apoptotic rates of A431 cells following treatment with gefitinib and/or CQ, flow cytometry with Annexin V/PI staining was performed. Significantly increased levels of Annexin V-positive A431 cells were identified in the combinatory treatment group compared with cells treated with gefitinib or CQ alone (Fig. 3B and C).

Furthermore, whether caspase-3 and PARP proteins serve important roles in the gefitinib + CQ-induced apoptosis of A431 cells was investigated via western blot analysis. Compared with the negative control, levels of the cleaved subunits of caspase-3 as well as cleaved PARP protein levels were increased in all treatment groups. In particular, the combination group (gefitinib + CQ) exhibited enhanced protein levels of cleaved PARP and cleaved caspase-3 (Fig. 3D). These results suggest that apoptosis in A431 cells is induced by co-treatment with CQ and gefitinib via caspase-dependent pathways.

CQ suppresses autophagy via inhibition of autophagosome degradation. Following pretreatment with CQ for 1 h, increased levels of LC3-II protein were observed in A431 cells treated with increasing concentrations of gefitinib for 12 h (Fig. 4A). LC3-II protein levels were increased in a time-independent manner, which suggested that CQ increased the number of autophagosomes by preventing fusion of lysosomes and autophagosomes, which can lead to autophagy inhibition (Fig. 4B) (21). To further investigate the effects of CQ on autophagic activity in A431 cells, basic autophagy activities exhibited by A431 cells were determined via MDC staining. Compared with the negative control, A431 cells treated with either gefitinib or CQ demonstrated weak fluorescence intensity in the cytosol; however, a number of bright foci were visualised in cells belonging to the combination group.
These results suggest that gefitinib activates the autophagy response in A431 cells and CQ blocks autophagy via inhibition of autophagosome degradation.

Discussion

In the present study, the pro-apoptotic role of gefitinib in cSCC cells was investigated and autophagy induced by treatment with gefitinib was revealed to represent a survival mechanism in cSCC cells. In addition, the results revealed that pro-survival autophagic flux may be blocked via treatment with CQ, which interferes with the fusion of autophagosomes with lysosomes. The results of the present study suggested that combinatorial usage of gefitinib with CQ may represent an effective therapeutic strategy for the treatment of patients with cSCC.

Preclinical data have demonstrated that EGFR has an important role in the carcinogenesis of cSCC (23), which resulted in the development of EGFR-targeting antibodies and TKIs, including gefitinib. When used as a neoadjuvant therapy, gefitinib has a 45.5% response rate and is well tolerated in patients with aggressive cSCC (24,25). Gefitinib has a therapeutic effect on patients suffering from non-small cell lung cancer (NSCLC) with EGFR mutations (26); however, the majority of patients exhibiting a response eventually develop acquired resistance to EGFR-TKIs (27). It has been well established that the therapeutic benefits of EGFR-targeting therapy may be suppressed by the requirement of autophagy for growth, survival and therapy resistance (28). The present study investigated the potential of autophagy inhibition, induced by CQ, for the enhancement of anti-proliferative effects of gefitinib in A431 cells.
Figure 3. Ge and CQ induce apoptosis via the caspase-dependent apoptosis pathway. (A) A431 cells were stained with acridine orange/ethidium bromide to determine levels of apoptosis and then observed under a fluorescence microscope. Magnification, x10. Scale bar, 500 µm. (B) Following treatment with 20 µM Ge and 188 µM CQ for 12 h, levels of apoptosis were analysed in A431 cells. (C) Statistical analysis of apoptosis levels in A431 cells in untreated, single drug treatment and combination treatment groups. Data are expressed as the mean ± standard deviation (n=3). ***P<0.001 vs. control group. ###P<0.001 vs. Ge treatment group. ####P<0.001 vs. CQ treatment group. (D) Protein expression levels of caspase-3, cleaved caspase-3, PARP and cleaved PARP. CQ, chloroquine diphosphate; Ge, gefitinib; PARP, poly-(ADP-ribose) polymerase; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Figure 4. Ge induces autophagy and CQ blocks autophagy in A431 cells. (A) A431 cells were treated with increasing concentrations of Ge for 12 h in the presence or absence of pre-treatment with CQ for 1 h. Expression levels of LC3-II were detected via western blotting. (B) A431 cells were treated with Ge for 3, 6, 9 or 12 h in the presence or absence of pre-treatment with CQ for 1 h. Expression levels of LC3-II were detected via western blotting. (C) A431 cells observed under a fluorescence microscope were stained with monodansylcadaverine to identify the formation of autophagic vacuoles. Magnification, x20. Scale bar, 500 µm. CQ, chloroquine diphosphate; Ge, gefitinib; LC3-II, microtubule associated protein 1 light chain 3β.
The results of the present study demonstrated that gefitinib and CQ inhibited the proliferation of A431 cells in a dose-dependent manner. Analysis performed using CompuSyn software revealed that combinatory treatment with gefitinib and CQ inhibited cell growth and enhanced synergistic drug interaction. Such drug combination methods allow for quantitative determination of drug interactions by determining CI values, in which CI<1, =1 and >1 indicate synergism, additive effect and antagonism, respectively (29). Combinatory treatment with gefitinib and CQ exhibited moderate synergistic effects in A431 cells, with CI values ranging from 0.742-0.989 for fraction affected (Fa)=0.543-0.962. Fa is commonly used to assess cell mortality following drug treatment, although this value does not demonstrate synergistic effects, which was evaluated by CI value (28). Drug synergism was also investigated using an isobologram, the results of which were previously revealed to be in agreement with Fa-CI plots (30). Synergistic effects were demonstrated by different dosages of drug combinations below their respective Fa isobe. DRI values are used to determine the effects of combinatory drug treatments (31). The DRI value of combinatory treatment with gefitinib and CQ was revealed to be >1 (1.654-2.328) in A431 cells, thus suggesting drug synergism. Furthermore, flow cytometric analysis and acridine orange/ethidium bromide staining revealed that CQ enhanced gefitinib-induced apoptosis, which was further demonstrated by increased expression levels of cleaved PARP and cleaved caspase-3 protein. Collectively, these results suggested that combinatory treatment with gefitinib and CQ synergistically induced apoptosis in A431 cells via the caspase-dependent apoptosis pathway.

The sensitivity of EGFR-targeting therapy is increased by inhibition of autophagy in NSCLC cells (32). The results of the present study demonstrated that suppressed levels of autophagy enhanced the levels of apoptosis in A431 cells. In conclusion, these results suggest that autophagy has a self-protective role in cell survival and contributes to drug resistance (33). LC3 is a marker for autophagy, and contains LC3-I and LC3-II (34). Cytosolic LC3-I is converted into membrane-bound LC3-II during the initiation of autophagy and thus LC3-II levels are associated with the number of autophagosomes (35). In the present study, gefitinib-induced autophagy in A431 cells was indicated by markedly increased levels of LC3-II in a dose-dependent manner. Furthermore, pre-treatment with CQ prior to treatment with gefitinib further increased LC3-II, which indicated autophagy was blocked by CQ. The results of the MDC staining demonstrated this effect. Therefore, the results of the present study suggested that autophagy induced by gefitinib regulates cytoprotective effects in A431 cells, and could be inhibited by CQ. However, the possible mechanisms of autophagy associated with this effect require further investigation. Numerous studies have demonstrated that increased levels of cytotoxicity associated with autophagy inhibition are exhibited by glioblastoma cells induced by vandetanib (36), by lung cancer cells induced by gefitinib and erlotinib (37), and by breast cells induced by gefitinib (38). However, limited studies have demonstrated the synergistic effect between autophagy inhibition and EGFR-targeting therapy, which was investigated in the present study. The results of the present study are notable, as the Chou-Talalay method regarding drug combination was used in the present study to quantitatively determine synergistic effects, which revealed that combinatory therapy of EGFR-targeting and autophagy-inhibition may represent a therapeutic strategy for patients with cSCC.

In conclusion, combinatory treatment using gefitinib and CQ on A431 cells exhibited a synergistic effect regarding increased levels of apoptosis. Autophagy, a cytoprotective effect associated with drug administration, was revealed to be inhibited by CQ, which subsequently enhanced gefitinib-mediated apoptosis via caspase-dependent pathways. Therefore, combinatory treatment using gefitinib and CQ may present a potential novel therapeutic strategy for the treatment of patients with cSCC. To further confirm the results of the present study, future studies should determine the underlying mechanisms associated with such effects. CQ represents a promising adjuvant approach for improving the efficacy of gefitinib for the treatment of patients with cSCC; however, this should be investigated further using in vivo preclinical models.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors’ contributions

JW, CW, XH, ZD and MZ conceived and designed the study. JW, CW, XH and ZD participated in implementation of the study. CW, XH and LZ assisted in collecting the data. JW, CW, XH and CY performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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