SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 18: 368-374, 2019

Gefitinib-mediated apoptosis is enhanced via inhibition of autophagy by chloroquine diphosphate in cutaneous squamous cell carcinoma cells

JIANYU WANG*, CHAOPENG WANG*, XIA HU*, CHANG YU, LIANG ZHOU, ZHENHUA DING and MEIJUAN ZHOU

Department of Radiation Medicine, School of Public Health, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

Received April 23, 2018; Accepted March 21, 2019

DOI: 10.3892/ol.2019.10308

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.

Correspondence to: Professor Meijuan Zhou, Department of Radiation Medicine, School of Public Health, Southern Medical University, 1023 Shatai South Road, Guangzhou, Guangdong 510515, P.R. China
E-mail: lkzmj@smu.edu.cn

*Contributed equally

Abbreviations: cSCC, cutaneous squamous cell carcinoma; EGFR, epidermal growth factor receptor; CQ, chloroquine diphosphate; IC_{50}, half-maximal inhibitory concentration; CI, combination index; DRI, dose reduction index; mAbs, monoclonal antibodies; TKIs, tyrosine kinase inhibitors; DMEM, Dulbecco's modified Eagle's medium; CCK-8, Cell Counting Kit-8; MDC, monodansylcadaverine; TBST, Tris-buffered saline-Tween 20; NSCLC, non-small cell lung cancer

Key words: cutaneous squamous cell carcinoma, autophagy, apoptosis, gefitinib, chloroquine diphosphate, synergism

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 EFGR 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 zalutunumab, target the extracellular portion of the receptor; however, TKIs, including gefitinib, erlotinib, lapatinib and afatinib, block the intracellular 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 tumour 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 autophagolysosome 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 with 5% CO_2 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 primary antibodies and dilutions were used in the present study: Microtubule associated protein 1 light chain 3-B (LC3B; cat. no. 3868S; Cell Signalling Technology, Inc., Danvers, MA, USA; 1:1,000), caspase-3 (cat. no. sc-7272; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:1,000), poly-(ADP-ribose) polymerase (PARP; cat. no. 9532S, Cell Signalling Technology, Inc.; 1:5,000), β-actin (cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Inc.; 1:1,000) and α-tubulin (cat. no. sc-5286; Santa Cruz Biotechnology; Inc.; 1:1,000). Secondary antibodies used in the present study were horseradish peroxidase (HRP)-tagged anti-mouse IgG (cat. no. 31430; 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 $8x10^3$ 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 (IC_{50}) 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 Cell-Quest 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 the 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 combinatory 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 combinatory 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

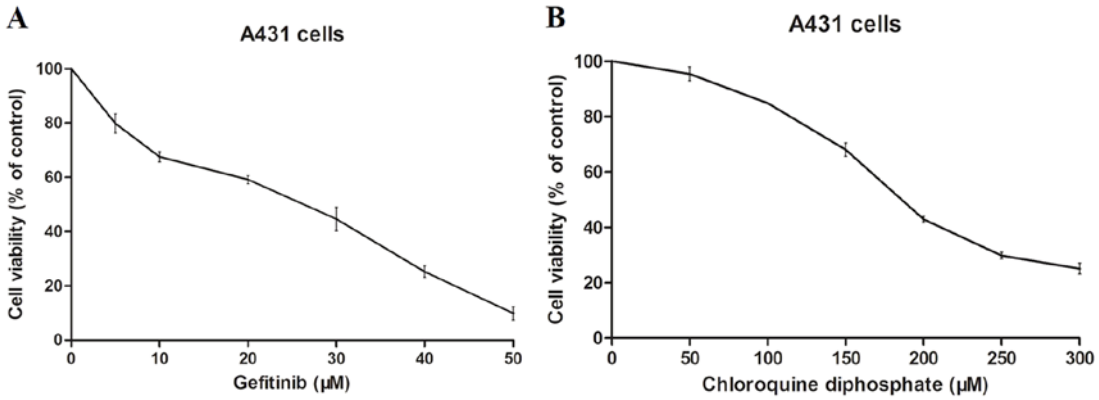
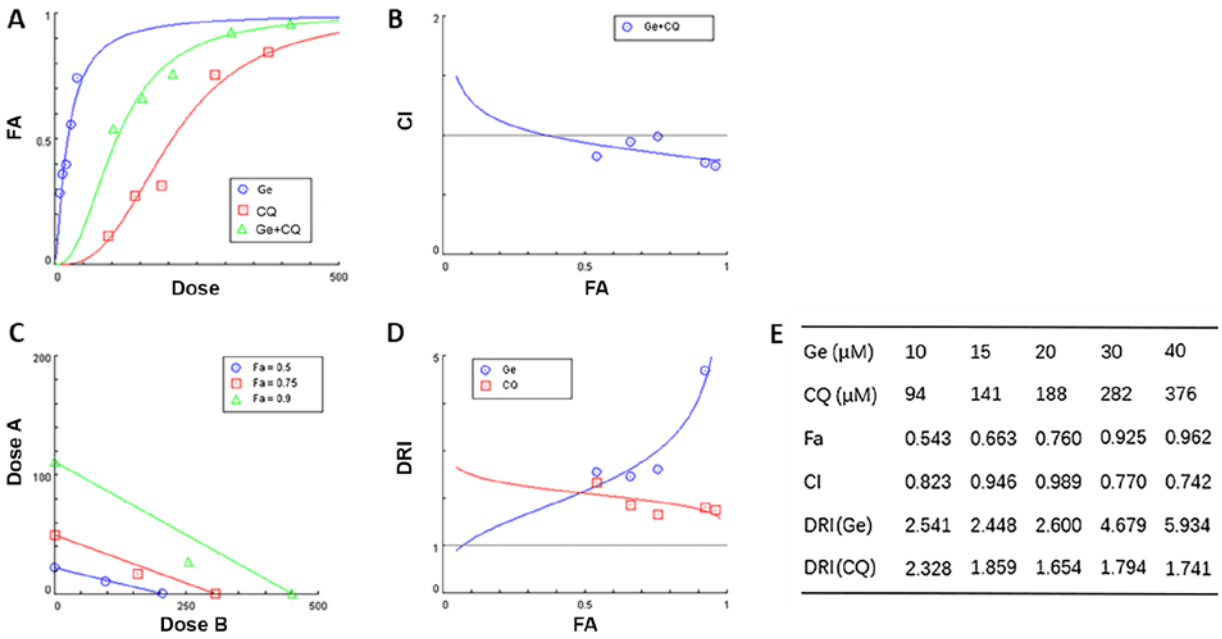


Figure 1. Cytotoxicity of gefitinib and chloroquine diphosphate. Cellular cytotoxicity in A431 cells treated with either (A) gefitinib or (B) chloroquine diphosphate in A431 cells was investigated via Cell Counting Kit-8 assays. Data are presented as the means ± standard deviation (n=3).



E

Ge (μM)	10	15	20	30	40
CQ (μM)	94	141	188	282	376
Fa	0.543	0.663	0.760	0.925	0.962
CI	0.823	0.946	0.989	0.770	0.742
DRI(Ge)	2.541	2.448	2.600	4.679	5.934
DRI(CQ)	2.328	1.859	1.654	1.794	1.741

Figure 2. CompuSyn analysis of cytotoxicity data was used to determine synergy, additivity and antagonism between Ge and CQ in A431 cells. (A) Dose-effect plots of Ge, CQ and Ge + CQ. (B) CI plots presenting CI values of <1 indicated synergism between Ge and CQ. (C) Isobolograms revealing effective doses required for inhibition at 50% (F_a 0.5), 75% (F_a 0.75) and 90% (F_a 0.9) for each individual drug. Synergism is demonstrated by the dose pair plotted as a point (symbol) below their respective F_a isobole or line. (D) DRI of Ge and CQ drug combinations are presented, and >1 DRI value indicated favourable drug combinations. (E) Data obtained via CompuSyn analysis. All data are representative of three independent experimental repeats. CI, combination index; DRI, dose reduction index; Ge, gefitinib; CQ, chloroquine diphosphate; F_a, fraction affected.

(Fig. 4C). 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.

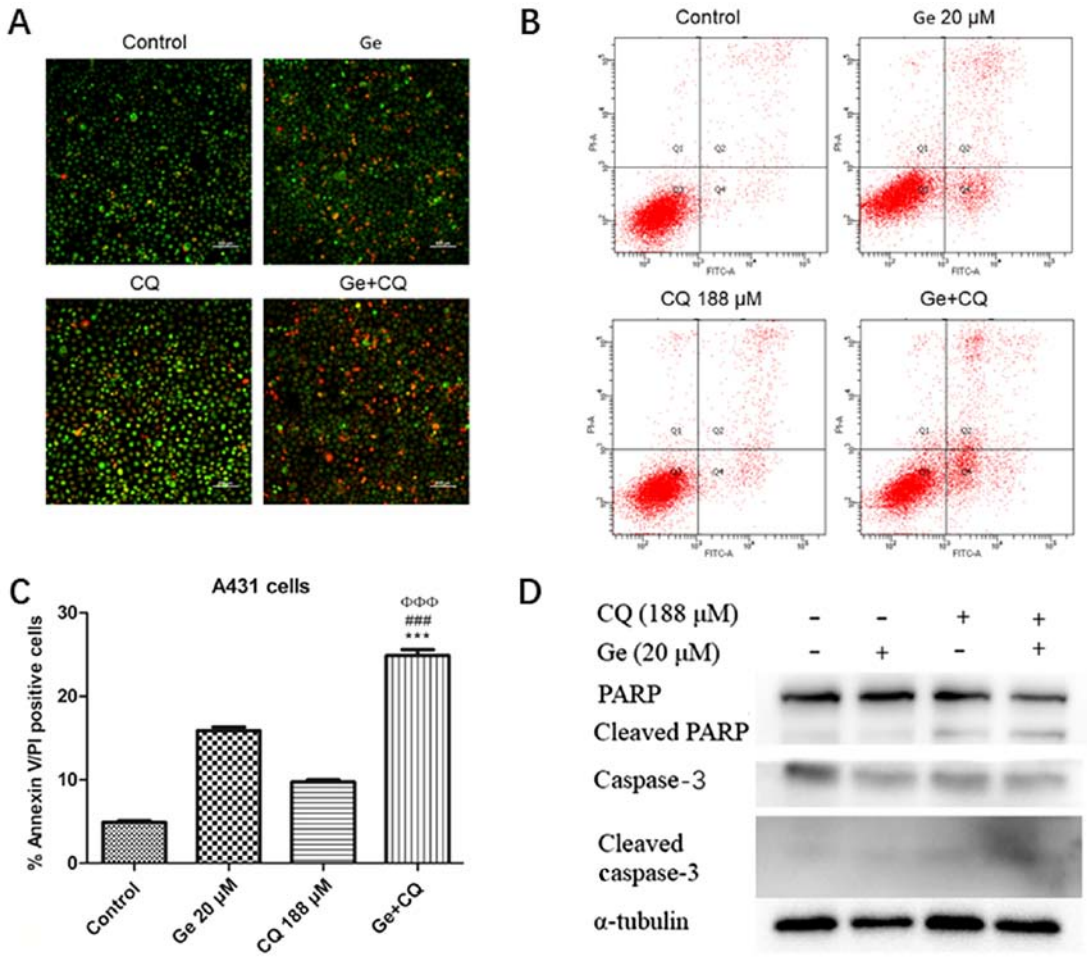


Figure 3. Ge and CQ induce apoptosis via the caspase-dependent apoptosis pathway. (A) A431 cells were stained with acridine orange/ethidium bromide to determine levels of apoptosis and then observed under a fluorescence microscope. Magnification, x10. Scale bar, 500 μm. (B) Following treatment with 20 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. $^{\Phi\Phi\Phi}$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.

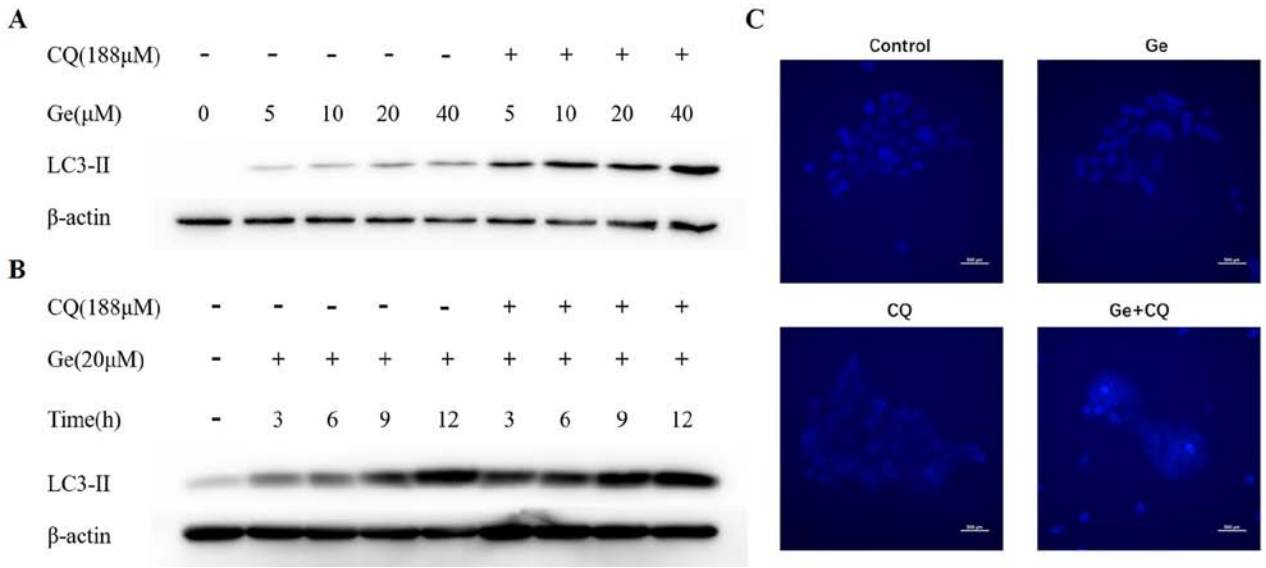


Figure 4. Ge induces autophagy and CQ blocks autophagy in A431 cells. (A) A431 cells were treated with increasing concentrations of Ge for 12 h in the presence or absence of pre-treatment with CQ for 1 h. Expression levels of LC3-II were detected via western blotting. (B) A431 cells were treated with 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 (F_a)=0.543-0.962. F_a 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 F_a-CI plots (30). Synergistic effects were demonstrated by different dosages of drug combinations below their respective F_a isobole. 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.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81472922).

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 and XH drafted the manuscript. JW, CW, XH and CY 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.

References

1. Stratigos A, Garbe C, Lebbe C, Malvehy J, del Marmol V, Pehamberger H, Peris K, Becker JC, Zalaudek I, Saiag P, *et al*: Diagnosis and treatment of invasive squamous cell carcinoma of the skin: European consensus-based interdisciplinary guideline. Eur J Cancer 51: 1989-2007, 2015.

2. Rogers HW, Weinstock MA, Harris AR, Hinckley MR, Feldman SR, Fleischer AB and Coldiron BM: Incidence estimate of nonmelanoma skin cancer in the United States, 2006. Arch Dermatol 146: 283-287, 2010.
3. de Vries E, Trakatelli M, Kalabalikis D, Ferrandiz L, Ruiz-de-Casas A, Moreno-Ramirez D, Sotiriadis D, Ioannides D, Aquilina S, Apap C, *et al*: Known and potential new risk factors for skin cancer in European populations: A multicentre case-control study. Br J Dermatol 167 (Suppl 2): S1-S13, 2012.
4. El-Abaseri TB, Fuhrman J, Trempus C, Shendrik I, Tennant RW and Hansen LA: Chemoprevention of UV light-induced skin tumorigenesis by inhibition of the epidermal growth factor receptor. Cancer Res 65: 3958-3965, 2005.
5. Hynes NE and Lane HA: ERBB receptors and cancer: The complexity of targeted inhibitors. Nat Rev Cancer 5: 341-354, 2005.
6. Sahu N and Grandis JR: New advances in molecular approaches to head and neck squamous cell carcinoma. Anticancer Drugs 22: 656-664, 2011.
7. Cañueto J, Cardeñoso E, García JL, Santos-Briz Á, Castellanos-Martín A, Fernández-López E, Blanco Gómez A, Pérez-Losada J and Román-Curto C: Epidermal growth factor receptor expression is associated with poor outcome in cutaneous squamous cell carcinoma. Br J Dermatol 176: 1279-1287, 2017.
8. Zheng Y, Su C, Zhao L and Shi Y: mAb MDR1-modified chitosan nanoparticles overcome acquired EGFR-TKI resistance through two potential therapeutic targets modulation of MDR1 and autophagy. J Nanobiotechnology 15: 66, 2017.
9. Huang S, Armstrong EA, Benavente S, Chinnaiyan P and Harari PM: Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): Combining anti-EGFR antibody with tyrosine kinase inhibitor. Cancer Res 64: 5355-5362, 2004.
10. Corkery B, Crown J, Clynes M and O'Donovan N: Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol 20: 862-867, 2009.
11. El Guerrab A, Bamdad M, Kwiatkowski F, Bignon YJ, Penault-Llorca F and Aubel C: Anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors as combination therapy for triple-negative breast cancer. Oncotarget 7: 73618-73637, 2016.
12. Tan X, Thapa N, Sun Y and Anderson RA: A kinase-independent role for EGF receptor in autophagy initiation. Cell 160: 145-160, 2015.
13. Mizushima N: The pleiotropic role of autophagy: From protein metabolism to bactericide. Cell Death Differ 12 (Suppl 2): S1535-S1541, 2005.
14. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, *et al*: Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12: 1-222, 2016.
15. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, *et al*: Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10: 51-64, 2006.
16. Claerhout S, Verschooten L, Van Kelst S, De Vos R, Proby C, Agostinis P and Garmyn M: Concomitant inhibition of AKT and autophagy is required for efficient cisplatin-induced apoptosis of metastatic skin carcinoma. Int J Cancer 127: 2790-2803, 2010.
17. Fung C, Chen X, Grandis JR and Duvvuri U: EGFR tyrosine kinase inhibition induces autophagy in cancer cells. Cancer Biol Ther 13: 1417-1424, 2012.
18. Jutten B, Keulers TG, Schaaf MB, Savelkouls K, Theys J, Span PN, Vooijs MA, Bussink J and Rouschop KM: EGFR overexpressing cells and tumors are dependent on autophagy for growth and survival. Radiother Oncol 108: 479-483, 2013.
19. Kang M, Lee KH, Lee HS, Jeong CW, Kwak C, Kim HH and Ku JH: Concurrent autophagy inhibition overcomes the resistance of epidermal growth factor receptor tyrosine kinase inhibitors in human bladder cancer cells. Int J Mol Sci 18, 2017.
20. Liu Z, He K, Ma Q, Yu Q, Liu C, Ndege I, Wang X and Yu Z: Autophagy inhibitor facilitates gefitinib sensitivity in vitro and in vivo by activating mitochondrial apoptosis in triple negative breast cancer. PLoS One 12: e0177694, 2017.
21. Iwai-Kanai E, Yuan H, Huang C, Sayen MR, Perry-Garza CN, Kim L and Gottlieb RA: A method to measure cardiac autophagic flux in vivo. Autophagy 4: 322-329, 2008.
22. Munafó DB and Colombo MI: A novel assay to study autophagy: Regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114: 3619-3629, 2001.
23. Uribe P and Gonzalez S: Epidermal growth factor receptor (EGFR) and squamous cell carcinoma of the skin: Molecular bases for EGFR-targeted therapy. Pathol Res Pract 207: 337-342, 2011.
24. Lewis CM, Glisson BS, Feng L, Wan F, Tang X, Wistuba II, El-Naggar AK, Rosenthal DI, Chambers MS, Lustig RA and Weber RS: A phase II study of gefitinib for aggressive cutaneous squamous cell carcinoma of the head and neck. Clin Cancer Res 18: 1435-1446, 2012.
25. William WN Jr, Feng L, Ferrarotto R, Ginsberg L, Kies M, Lippman S, Glisson B and Kim ES: Gefitinib for patients with incurable cutaneous squamous cell carcinoma: A single-arm phase II clinical trial. J Am Acad Dermatol 77: 1110-1113.e2, 2017.
26. Ono M and Kuwano M: Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clin Cancer Res 12: 7242-7251, 2006.
27. Huang MH, Lee JH, Chang YJ, Tsai HH, Lin YL, Lin AM and Yang JC: MEK inhibitors reverse resistance in epidermal growth factor receptor mutation lung cancer cells with acquired resistance to gefitinib. Mol Oncol 7: 112-120, 2013.
28. Jutten B and Rouschop KM: EGFR signaling and autophagy dependence for growth, survival, and therapy resistance. Cell Cycle 13: 42-51, 2014.
29. Chou TC: Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70: 440-446, 2010.
30. Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621-681, 2006.
31. Chou TC and Talalay P: Generalized equations for the analysis of inhibitions of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur J Biochem 115: 207-216, 1981.
32. Li YY, Lam SK, Mak JC, Zheng CY and Ho JC: Erlotinib-induced autophagy in epidermal growth factor receptor mutated non-small cell lung cancer. Lung Cancer 81: 354-361, 2013.
33. Galluzzi L, Pietrocola F, Levine B and Kroemer G: Metabolic control of autophagy. Cell 159: 1263-1276, 2014.
34. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19: 5720-5728, 2000.
35. Behrends C, Sowa ME, Gygi SP and Harper JW: Network organization of the human autophagy system. Nature 466: 68-76, 2010.
36. Shen J, Zheng H, Ruan J, Fang W, Li A, Tian G, Niu X, Luo S and Zhao P: Autophagy inhibition induces enhanced proapoptotic effects of ZD6474 in glioblastoma. Br J Cancer 109: 164-171, 2013.
37. Han W, Pan H, Chen Y, Sun J, Wang Y, Li J, Ge W, Feng L, Lin X, Wang X, *et al*: EGFR tyrosine kinase inhibitors activate autophagy as a cytoprotective response in human lung cancer cells. PLoS One 6: e18691, 2011.
38. Dragowska WH, Weppler SA, Wang JC, Wong LY, Kapanen AI, Rawji JS, Warburton C, Qadir MA, Donohue E, Roberge M, *et al*: Induction of autophagy is an early response to gefitinib and a potential therapeutic target in breast cancer. PLoS One 8: e76503, 2013.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.