Cancer cell-specific anticancer effects of *Coptis chinensis* on gefitinib-resistant lung cancer cells are mediated through the suppression of Mcl-1 and Bcl-2

JAE HWAN KIM[1,2*], EUN SUN KO[1,2*], DASOM KIM[1,2], SEONG-HEE PARK[1,2], EUN-JUNG KIM[3], JINKYUNG RHO[4], HYEMIN SEO[5], MIN JUNG KIM[5], WOONG MO YANG[6], IN JIN HA[7], MYUNG-JIN PARK[8] and JI-YUN LEE[1]

Departments of [1]Pathology, and [2]Biomedical Science, Korea University College of Medicine, Seoul 02841; [3]Department of Acupuncture and Moxibustion, College of Oriental Medicine, Dongguk University, Gyeongju-si, Gyeongsangbuk-do 38066; [4]Asan Institute for Life Sciences, Asan Medical Center, College of Medicine, University of Ulsan, Seoul 05505; [5]Department of Biological Sciences, Sookmyung Women's University, Seoul 04310; [6]College of Korean Medicine, Kyung Hee University; [7]Korean Medicine Clinical Trial Center (K-CTC), Kyung Hee University Korean Medicine Hospital, Seoul 02447; [8]Division of Radiation Cancer Research, Research Center for Radio-Senescence, Korea Institute of Radiological and Medical Sciences, Seoul 02841, Republic of Korea

Received June 17, 2019; Accepted February 14, 2020

DOI: 10.3892/ijo.2020.5025

Abstract. The epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI), gefitinib, is an effective therapeutic drug used in the treatment of non-small cell lung cancers (NSCLCs) harboring EGFR mutations. However, acquired resistance significantly limits the efficacy of EGFR-TKIs and consequently, the current chemotherapeutic strategies for NSCLCs. It is, therefore, necessary to overcome this resistance. In the present study, the anticancer potential of natural extracts of *Coptis chinensis* (ECC) against gefitinib-resistant (GR) NSCLC cells were investigated *in vitro* and *in vivo*. ECC inhibited the viability, migration and invasion, and effectively induced the apoptosis of GR cells. These effects were associated with the suppression of EGFR/AKT signaling and the expression of anti-apoptotic proteins, Mcl-1 and Bcl-2, which were overexpressed in GR NSCLC cells. Combination treatment with ECC and gefitinib enhanced the sensitivity of GR cells to gefitinib *in vitro*, but not *in vivo*. However, ECC increased the survival of individual zebrafish without affecting the anticancer effect to cancer cells *in vivo*, which indicated a specific cytotoxic effect of ECC on cancer cells, but not on normal cells; this is an important property for the development of novel anticancer drugs. On the whole, the findings of the present study indicate the potential of ECC for use in the treatment of NSCLC, particularly in combination with EGFR-TKI therapy, in EGFR-TKI-resistant cancers.

Correspondence to: Professor Ji-Yun Lee, Department of Pathology, Korea University College of Medicine, 73 Goryeodae-ro, Seongbuk-gu, Seoul 02841, Republic of Korea
E-mail: jiyun-lee@korea.ac.kr

Dr Myung-Jin Park, Division of Radiation Cancer Research, Research Center for Radio-Senescence, Korea Institute of Radiological and Medical Sciences, 73 Goryeodae-ro, Seongbuk-gu, Seoul 02841, Republic of Korea
E-mail: mjpark@kirams.re.kr

*Contributed equally

Key words: Coptis chinensis, epidermal growth factor receptor, gefitinib, epidermal growth factor receptor-tyrosine kinase inhibitor resistance, apoptosis

Introduction

The ability to evade apoptosis is one of the hallmarks of cancer and is a crucial property of cancer cells that confers them resistance to chemotherapeutic agents (1,2). Understanding apoptotic resistance may assist in the development of strategies with which to restore the sensitivity of cancer cells to apoptosis and, ultimately, may improve the efficacy of cancer therapy. In lung cancer, various epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) resistance mechanisms have been identified, such as the second-site EGFR mutation, T790M, the activation of the bypass pathways, MET and AXL, and histological transformation, and several efforts have been made to overcome these resistance mechanisms (3,4). A common apoptosis-associated EGFR-TKI mechanism in lung cancer is an intrinsic deletion polymorphism in the gene encoding BIM, although the findings regarding this are contradictory (5-7). BIM is a pro-apoptotic member of the Bcl-2 family and plays an essential role in the induction of cell apoptosis and tumor metastasis (2). The upregulation of BIM is required for apoptosis induced by EGFR and EGFR-TKIs in tumors harboring EGFR mutations (8). Consequently,

BIM has become the focus of attention as a potential target for cancer chemotherapy. Furthermore, the overexpression of anti-apoptotic Bcl-2 family proteins, including Mcl-1 and Bcl-2, has been investigated and has been identified to be associated with chemoresistance and the prognosis of various types of cancer, including lung cancer (9-12); however, there are a limited number of studies on EGFR-TKI-resistant lung cancer.

The herb *Coptis chinensis* (known as goldthread; CC) is widely used in Traditional Chinese medicine; moreover, its alkaloid component, berberine, has been studied for its multiple pharmacological activities, including anti-infectious, anti-inflammatory and anticancer effects (13). In addition, efforts have been made to examine the potential therapeutic and biological functions of CC, not as a single compound, but as multi-compounds, for cancer treatment. CC has been shown to exert an anticancer effect through the downregulation of signal transducer and activator of transcription (STAT)2 phosphorylation by reducing the level of histone deacetylase 2 (HDAC2) in glioma cells and inhibiting hepatocellular carcinoma cell growth through non-steroidal anti-inflammatory drug (NSAID) activated gene (NAG-1) activation (14,15). In non-small cell lung cancer (NSCLC) cells, CC has been shown to inhibit growth and metastasis, and to induce cell apoptosis (16). However, neither its effects on EGFR-TKI resistant lung cancer nor its efficacy in combination with gefitinib have been elucidated to date, at least to the best of our knowledge.

Therefore, the present study examined the expression of Mcl-1 and Bcl-2 in order to determine the effects of the extract of CC (ECC) on apoptosis. The anticancer effects of ECC, as well as combination treatment with ECC and gefitinib on gefitinib-resistant (GR) NSCLC cells (PC9GR, A549GR and HCC827GR) were also investigated.

Materials and methods

Cell cultures and reagents. BEAS-2B, and the GR human lung cancer cell lines, PC9GR and A549GR, were gifts from Dr J.K. Rho, Ulsan University, Asan Hospital. The HCC827GRKU cell line was established from HCC827 cells treated with 2 μM gefitinib for >6 months (data not shown). All cell lines were grown in RPMI (Welgene, Inc.) supplemented with 10% fetal bovine serum (Welgene, Inc.) and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 for all the experiments. Gefitinib was purchased from Selleck Chemicals and berberine was purchased from Sigma-Aldrich; Merck KGaA. Air-dried roots of CC were purchased from Dongguk University, Ilsan Korean Medicine Hospital. CC (10 g) was extracted in 100 ml distilled water at room temperature. After 24 h, the solution was heated to 90˚C for 4 h. The extract was then filtered, evaporated and lyophilized (yield, 12.6%). The lyophilized extract of CC (ECC) was stored at -20˚C until use. The identification of chemical components in ECC was performed by ultra-performance liquid chromatography (UPLC)-quadrupole time-of-flight (QTOF) (Data S1, Fig. S1 and Table SI). ECC was re-dissolved in RPMI to a concentration of 1,000 μg/ml for the *in vitro* experiments and in DMSO (Daejunga) to a concentration of 6 mg/ml for the *in vivo* experiments.

Cell viability assays. Cell viability was measured by MTT assay. Briefly, $1x10^3$ cells per well were seeded in 96-well culture plates overnight and, subsequently incubated with or without the relevant treatments of ECC, or berberine. After 72 h, 50 μl MTT solution (0.5 mg/ml, Sigma-Aldrich; Merck KGaA) were added to each well. Following incubation at 37˚C for a further 4 h, the MTT solution was discarded and DMSO was added. The absorbance at 750 nm was measured using a microplate reader (SpectraMax Plus 384, Molecular Devices, LLC). The fraction affected (Fa) and combination index (CI) values were calculated using CompuSyn (www.combosyn.com). CI values of <1, 1, and >1 indicated synergism, additive effects and antagonism, respectively. Cell viability assay for the co-treatment was performed with selected concentrations of of gefitinib (PC9GR and HCC827GRKU cells, 1 μM; A549GR cells, 2 μM) and ECC (PC9GR and HCC827GRKU cells, 10 μg/ml; A549GR cells, 5 μg/ml) for 72 h based on the CI values. The results were representative of a minimum of 3 independent experiments, and the error bars represent the standard deviation (SD).

Transwell invasion assays. The invasiveness of the tumor cells was assessed via an invasion assay in Transwell chambers comprising a Transwell membrane (8 μm pore size, 6.5 mm in diameter, Corning Life Science, Inc.) coated with Matrigel (100 μg/ml, 10 μl/well). The cells ($1x10^5$) were seeded in the upper chambers in the presence of the indicated concentrations (PC9GR cells: 0, 30 and 50 μg/ml; HCC827GRKU cells: 0 and 30 μg/ml) of ECC. The lower chambers of the Transwell plate were filled with RPMI with 10% FBS The cells were fixed with 70% ethanol for 10 min, stained with hematoxylin and eosin for 5 min at room temperature, and counted under a light microscope (Olympus-IX71, Olympus Corp.) following incubation for 24 h.

Cell migration assay. Cell migration was assessed using a wound-healing assay. The cells ($5x10^5$) were seeded in 6-well plates and incubated at 37˚C for 24 h. After the cell monolayer was scraped with a sterile micropipette tip, the wells were washed several times with phosphate-buffered saline (PBS) and cultured with the designated concentrations (PC9GR cells: 0, 30 and 50 μg/ml; A549GR cells: 0, 10 and 20 μg/ml; HCC827GRKU cells: 0 and 30 μg/ml) of ECC. The first image of each scratch from 4 independent areas was acquired at time zero. The image of each scratch at the same location was captured under a light microscope (Olympus-IX71, Olympus Corp) after the indicated incubation times (0, 24 and 48 h). The healed area was measured from the captured images using Image J software (Ver. 1.52n, NIH).

Western blot analysis. Cell were lysed with ice-cold TNN buffer (1 M Tris-Cl pH 7.4, 0.5% NP40, 5 M NaCl., 0.5 M EDTA pH 8.0) at 4˚C for overnight. Cell lysates were centrifuged at 16,100 x g for 15 min and the supernatants were used as total cellular protein extracts. The protein concentrations were determined by Bradford assay (Microplate reader, model-680, Bio-rad). Protein denaturation (20 μg/lane) was carried out by sodium dodecyl sulfate (SDS) and mercaptoethanol loading and electrophoresed on a 12% acrylamide gel (this excluded caspase-3 which was electrophoresed on a 15% acrylamide gel).

This was followed by transfer onto nitrocellulose membranes (GE Healthcare Life Science, Inc.). The membranes were blocked with 5% non-fat dry milk (SK1400.500, BioShop) in TBST (247 mM Tris, 1.37 M NaCl, 27 mM KCl, 1% Tween-20, pH 7.6) at room temperature for 1 h. These membranes were, subsequently, probed with the indicated primary antibodies at 4˚C for overnight and incubated with the appropriate goat anti-mouse IgG (1:5,000, sc-2005, Santa Cruz Biotechnology, Inc.) or goat anti-rabbit IgG (1:5,000, sc-2004, Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Secondary antibodies were conjugated with horseradish peroxidase prior to signal detection using the enhanced chemiluminescence system (Translab) in accordance with the manufacturer's instructions. The primary antibodies (dilution, cat. no.) against EGFR (1:1,000, #2232), AKT (1:1,000, #4691), p-AKT (1:1,000, #4691), caspase-3 (1:1,000, #9662) and poly(ADP-ribose) polymerase (PARP) (1:1,000, #9542) were purchased from Cell Signaling Technology, Inc. The antibodies against p-EGFR (1:1,000, sc-101668), MET (1:1,000, sc-161), Bcl-2 (1:1,000, sc-492), Mcl-1 (1:1,000, sc-819), Bcl-xL (1:1,000, sc-7195) and β-actin (1:20,000, sc-47778) were purchased from Santa Cruz Biotechnology, Inc.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized from the total RNA using a reverse transcription kit (LaboPass, Cosmo Genetech) in accordance with the manufacturer's instructions. qPCR was conducted using gene-specific primers with SYBR-Green Q Master (LaboPass) on an ABI 7500 Real Time PCR System (Applied Biosystems). The following PCR primers were used: *Bcl-2* sense, 5'-AAGGGGGAAACACCAGAATC-3' and antisense, 5'-ATCCTTCCCAGAGGAAAAGC-3'; *Mcl-1* sense, 5'-TGC TGGAGTAGGAGCTGGTT-3' and antisense, 5'-CCTCTT GCCACTTGCTTTTC-3'; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* sense, 5'-GCCATCGTCACCAAC TGGGAC-3' and antisense, 5'-CGATTTCCCGCTCGGCCG TGG-3'. The PCR thermocycling conditions consisted of 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec and 62˚C for 30 sec. The Ct values of the target genes were normalized to those of an endogenous reference gene (*GAPDH*) using the ΔΔCq method (17). Each gene was analyzed in triplicate in 2 independent experiments.

Cell cycle analysis. GR cells were harvested following treatment with ECC (PC9GR cells, 50 μg/ml; A549GR cells, 30 μg/ml) for the indicated time periods (0, 24 and 48 h) and dissociated into single cells. The cells were fixed with 95% ethanol, incubated at -20˚C for at least 1 h, and washed with PBS. The cells were then resuspended in PBS with 0.1 mg/ml RNase A, 50 mg/ml propidium iodide (PI), and 0.05% Triton X-100 for 15 min at room temperature in the dark and washed with PBS. The stained samples were analyzed using a FACS Canto 2 (BD Biosciences) within 1 h of staining. All experiments were performed in triplicate.

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. Cells were seeded on a coverslip with complete medium and incubated with or without the indicated concentrations (PC9GR cells, 50 μg/ml; A549GR cells, 30 μg/ml) of ECC. Following incubation at 37˚C for 24 and 48 h, the cells were fixed with 4% paraformaldehyde for 25 min at 4˚C and washed twice with PBS at room temperature. The cells were then permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min and washed twice with PBS. TUNEL assay of the nuclei was performed, and the labeled cells were viewed under a fluorescent microscope (Olympus-IX71, Olympus Corp.), as described in the manufacturer's protocol (DeadEnd™ Fluorometric TUNEL System; Promega).

In vivo zebrafish tumor model. Zebrafish (*Danio rerio*) and embryos were bred and maintained according to standard procedures. All animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation of the Sookmyung Women's University and performed as previously described (18). Approximately 50 fluorescent cell tracker CM-Dil-labeled HCC827 or HCC827GRKU cells were injected into the yolk sac of each zebrafish embryo (100 embryos for each treatment), after which the embryos were maintained at 33˚C and drug treatments were administered every 24 h for 4 days. Fluorescence image acquisition was performed using a Zeiss LSM700 confocal microscope (Carl Zeiss AG). The area penetrated by the CM-Dil-labeled cancer cells was quantified using ImageJ software (ver. 1.52n, NIH) and normalized to the cancer cells (100%) in non-treated zebrafish embryos for each group.

Dose selection of ECC. For the *in vitro* assay, the IC_{50} value of 3 days for the PC9GR (32.73 μg/ml) and A549GR (20 μg/ml) cells and an approximately 1.5-fold higher concentration than the IC_{50} value of 3 days were selected. For the HCC827GRKU, one concentration was selected, which was approximately 1.5-fold higher than the IC_{50} value of 3 days. For the *in vivo* assay, the toxicity of ECC was tested on zebrafish embryo development at a series of concentrations of ECC (0.1, 1 and 5 μl/ml); the embryos were observed until 7 days post-fertilization (dpf) and the concentration which did not affect the survival of the zebrafish was selected. The zebrafish embryos died from overall necrosis, a terminated heart beat and no movement from mechanical stimulation at 4 dpf following treatment with 5 μl/ml of ECC, but survived and developed normally following treatment with 0.1 and 1 μl/ml of ECC. Subsequently, further tests were performed to select the ECC concentration which was most effective against cancer cells from a series of ECC concentrations (0.1, 0.2 and 1.0 μl/ml), which was 0.2 μl/ml.

Statistical analysis. The experiments were repeated at least twice. The values are expressed as the means ± standard deviation and were compared using a two-tailed Student's t-test or ANOVA. If the P-value obtained by one-way ANOVA was <0.05, P-values between the groups were compared with a post hoc test, such as the Bonferroni and Tukey's HSD. A value of $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

ECC inhibits the viability and mobility of the GR NSCLC cell lines, PC9GR, A549GR and HCC827GR. Given that, as

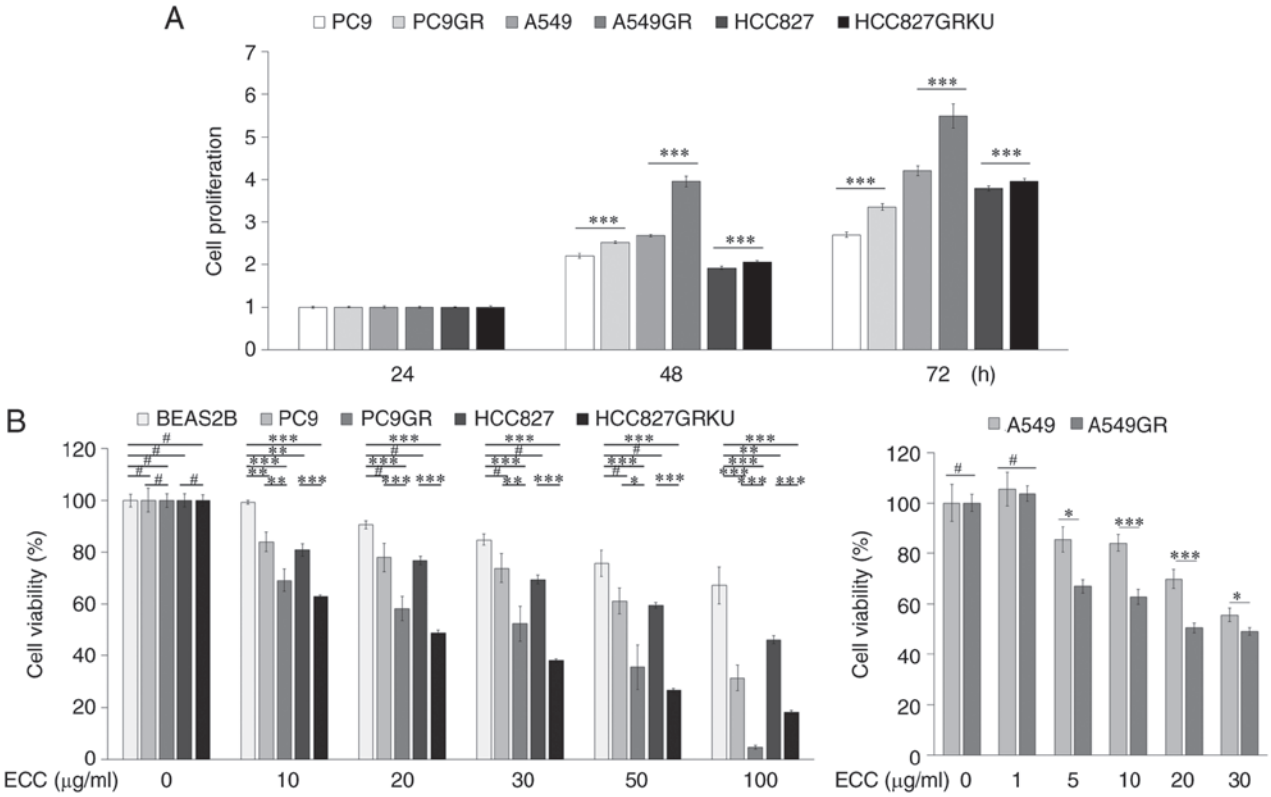

Figure 1. ECC inhibits the viability of GR cell lines (PC9GR, A549GR and HCC827GRKU), and their parental cells (PC9, A549 and HCC827) with minimal cytotoxic effects on BEAS-2B cells. (A) Proliferation of GR cells increased in comparison with their parental cells, as determined by MTT assay. (B) Cell viability was determined by MTT assay following treatment with ECC for 3 days and the GR cells were more sensitive to ECC than their parental cells. The results shown are the means ± SD of triplicate experiments. *P<0.05, **P<0.01 and ***P<0.001; the hash symbol (#) indicates that there were no significant differences. ECC, Extract of *Coptis chinensis*; GR, gefitinib-resistant.

previously demonstrated, the GR cell lines, PC9GR, A549GR and HCC827GR, exhibit an enhanced viability upon gefitinib treatment (18-20) and in this study, were found to proliferate more rapidly than their parental cells (Fig. 1A), an MTT assay was performed on these GR NSCLC cells, as well as on their parental cells PC9, A549 and HCC827 and the normal bronchus cell line, BEAS-2B, to examine the effects of ECC on cell viability. The effects of berberine (Data S1), a known alkaloid extracted from CC, on cell viability and cytotoxicity was examined using an MTT assay in order to compare its effects to those of ECC, which is a multi-compound formulation. ECC suppressed the viability of the GR cells more effectively than that of the parental cells and exerted minimal cytotoxic effects on the BEAS-2B cells (Fig. 1B and Table I). However, berberine was less toxic to the PC9GR cells than the PC9 cells, and exerted significant cytotoxic effects on the BEAS-2B cells; moreover, the BEAS-2B cells were even more sensitive to berberine than the HCC827 lung cancer cells (Fig. S2). The effects of ECC on the migratory and the invasive potential of the PC9GR, A549GR and HCC827GR cells were examined using *in vitro* migration and invasion assays. Treatment with ECC inhibited the migration and invasion of the GR cells in a dose- and time-dependent manner (Fig. 2); however, a limitation of the present study should be stated here in that 10% FBS may have affected cell proliferation. The invasion assay could not be performed for the A549GR cells, as the cells do not attach effectively on Matrigel. Collectively, these results revealed that ECC exerted anticancer effects on the GR cells.

ECC induces the apoptosis of GR NSCLC cells (PC9GR, and A549GR cells). To elucidate the mechanisms through which ECC affects GR cell viability, cell cycle and apoptosis analyses were performed using PI-stained cells through FACS analysis and TUNEL assay. The distribution of GR cells in the cell cycle phase was analyzed following treatment with the indicated concentrations of ECC for 24 and 48 h. ECC treatment increased the percentage of GR cells in the sub-G1 phase (i.e., dead cells) in a time-dependent manner (Fig. 3A-C). Apoptosis induced by ECC was confirmed by TUNEL assay, which revealed an increase in the number of TUNEL-positive cells upon ECC treatment (Fig. 3D). Thus, these data indicated that the anticancer effects of ECC on GR cells resulted from the induction of cell cycle arrest and cell death.

ECC suppresses the EGFR-AKT pathway and the expression of the anti-apoptotic proteins, Mcl-1 and Bcl-2. Increased cell survival owing to the impairment of an essential pathway for EGFR-TKI-mediated apoptosis has been suggested as a mechanism responsible for resistance to EGFR-TKIs. To investigate this pathway in GR cells, the expression of the EGFR pathway and anti-apoptotic proteins was examined in GR cells and compared with that in their parental cells. The expression of AKT/p-AKT and the anti-apoptotic proteins, Mcl-1 and/or Bcl-2, was increased in the GR cells (Figs. 4A and B, and S3A). As ECC exerted anti-survival and pro-apoptotic effects, the effects of ECC on the expression of AKT/p-AKT, Mcl-1 and Bcl-2 were then examined.

Table I. IC_{50} values of ECC, gefitinib and berberine in GR and parental cells.

Treatment	Cells lines and IC_{50} values						
	PC9	PC9GR	A549	A549GR	HCC827	HCC827GRKU	BEAS-2B
Gefitinib (μM)	0.008	8.79	15.34	18.65	0.01	10	N/A
ECC (μg/ml)	69.80	32.73	30	20	85.33	19.07	178.08
Berberine (μM)	2.81	7.73	13.99	<1	48.1	13.3	33.01

ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

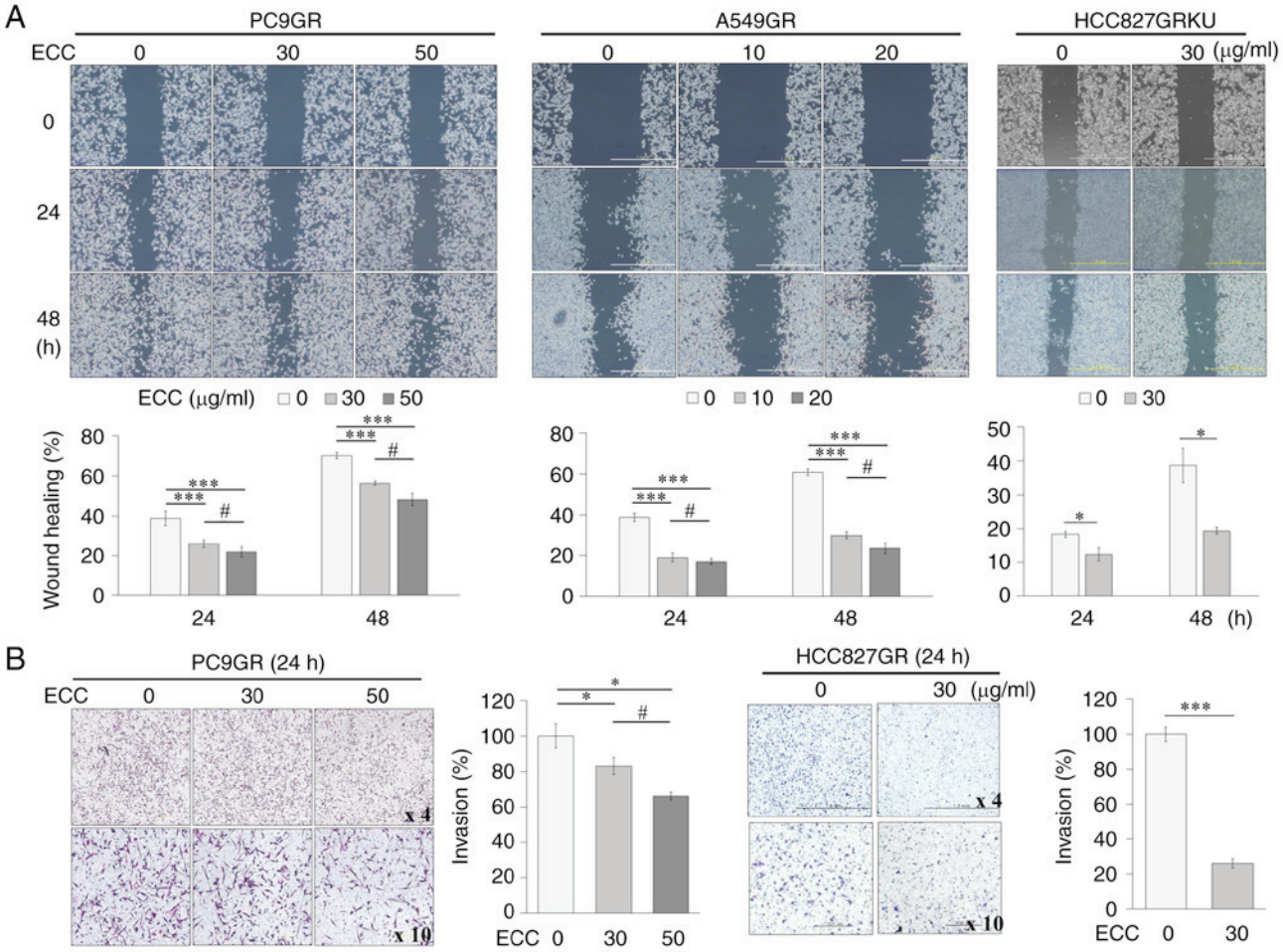

Figure 2. ECC inhibits the migration and invasion of GR cells (PC9GR, A549GR and HCC827GRKU). (A) Representative images of the scratched areas of the cultures of cells treated with the indicated concentrations of ECC collected at the indicated time after wounding with a pipette tip. (B) Representative images of cells that migrated through the Transwell and were stained with hematoxylin and eosin. The results are shown as the means ± SD of triplicate experiments. *P<0.05, and ***P<0.001; the hash symbol (#) indicates that there were no significant differences. ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

ECC treatment resulted in the suppression of the expression of these molecules (Figs. 4C and S3B). The effects of ECC on the expression of caspase-3 and PARP, which act as Mcl-1/Bcl-2 downstream effectors in the apoptotic pathway in GR cells were then further examined. The expression of Mcl-1 and Bcl-2 was decreased, and the cleaved forms of caspase-3 and PARP were increased in a time- and dose-dependent manner (Fig. 4C). The suppression of the expression of Mcl-1 and Bcl-2 by ECC was confirmed by RT-qPCR (Figs. 4D and S3C).

ECC synergistically enhances the activity of gefitinib in GR NSCLC cells in vitro. The ability of ECC to enhance the effects of gefitinib on GR cells was evaluated by MTT assay using cells treated with a combination of ECC and gefitinib. Combination treatment reduced GR cell viability in comparison to treatment with gefitinib or ECC alone (Fig. 5A). To elucidate the mechanisms through which ECC restores the antitumor activities of EGFR-TKIs, the activities of EGFR and its downstream molecule, AKT, as well as the anti-apoptotic proteins Bcl-2 and Mcl-1, were examined in GR

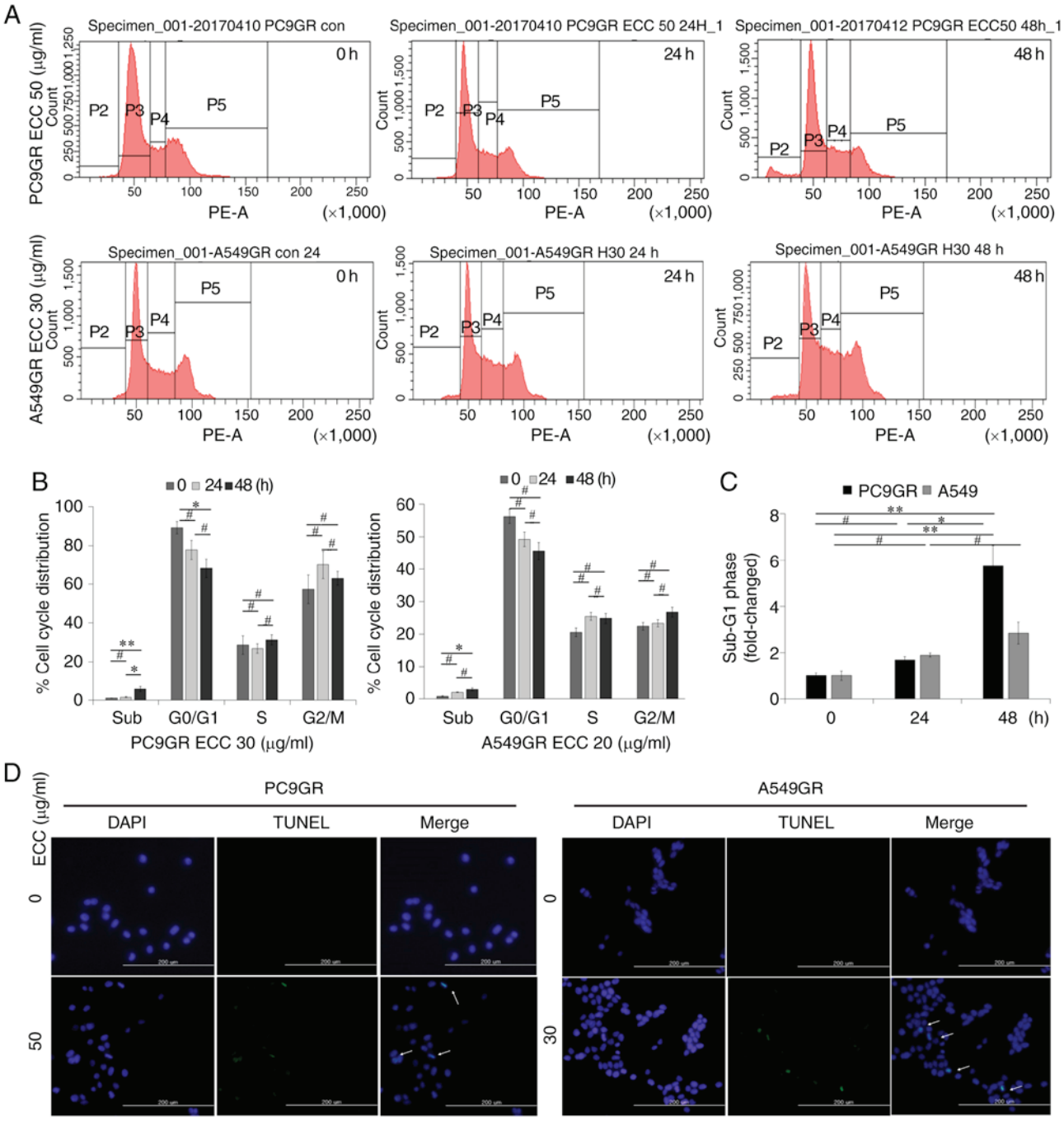

Figure 3. ECC induces the death of GR cell lines (PC9GR and A549GR). (A-C) Cells were treated with the indicated concentrations of ECC for the indicated periods of time. The cell cycle distribution of the harvested cells was analyzed by flow cytometry. Representative (A) histograms and (B) quantification of the analysis of cells in G1/G0, S, G2/M and (C) sub-G1 fractions phases shown in the figure were measured by FACS analysis. (D) Representative images of the immunocytochemistry of treated and untreated GR cells (left panel) examined by TUNEL assay after 24 h. TUNEL-positive nuclei are indicated with white arrows. The nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) staining. Magnification, x200. The results are the means ± SD of triplicate experiments. *P<0.05, and **P<0.01; the hash symbol (#) indicates that there were no significant differences. ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

cells. As expected, combination treatment resulted in the most effective inhibitory effects (Fig. 5B). It should be noted that in the PC9GR cells, combination treatment only decreased Bcl-2 expression at 48 h, not at 24 h (Fig. 5B). The reasons for this are not clear. In addition, whether the combination treatment was able to enhance the inhibitory effects gefitinib on cell viability through a synergistic effect was examined. This was determined by the Fa-CI plot (Chu-Talalay Plot; www.combosyn.com) median effect analysis, which revealed that the combination index (CI) was smaller than 1 (Fig. 5C) (21), indicating synergistic growth inhibition of the GR cells by this treatment combination.

Cancer cell-specific/sensitive toxicity of ECC in vivo. The suppression of tumorigenicity *in vivo* by ECC treatment in lung cancer cells was examined in xenograft zebrafish models. CM-Dil-labeled HCC827 or HCC827GRKU cells (red) were grafted into *Tg(flk1:EGFP)* zebrafish embryos

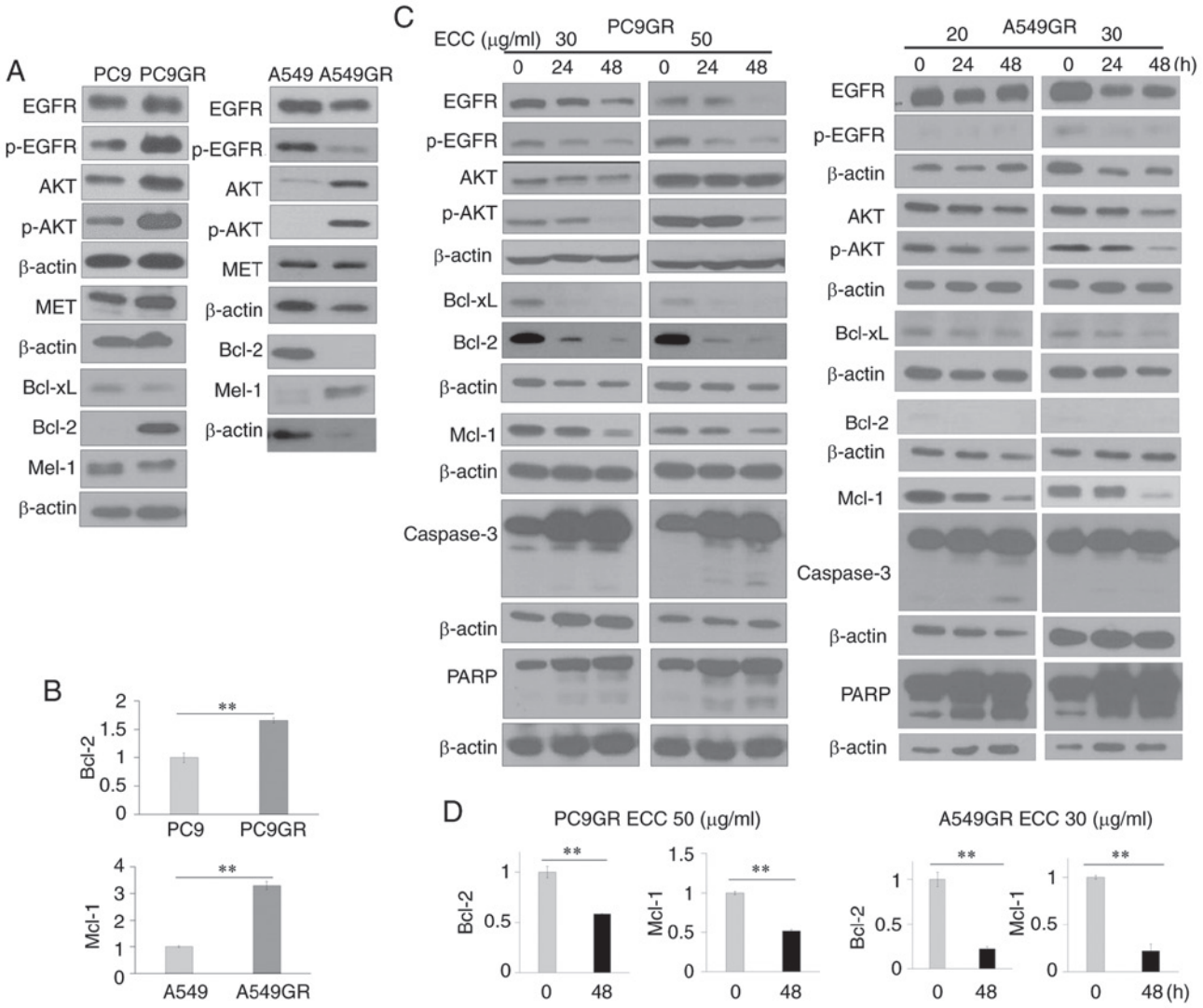

Figure 4. ECC suppresses the expression of EGFR/AKT and apoptosis-related signalling. (A) Anti-apoptotic proteins Bcl-2 or Mcl-1 were overexpressed in GR cells compared with their parental cells as examined by western blot analysis. (B) Higher expression of Bcl-2 in PC9GR than PC9 and Mcl-1 in A549GR than in A549 cells was confirmed by RT-qPCR. (C) ECC suppressed EGFR/AKT signalling and the anti-apoptotic proteins Bcl-2 and Mcl-1, resulting in increased cleaved caspase-3 and PARP expression in GR cells, as determined by western blot analysis. (D) Suppression of Bcl-2 and Mcl-1 by ECC was confirmed by RT-qPCR. GR cells were treated with ECC with the indicated concentrations for the indicated periods of time. The cells were harvested, and the indicated protein or RNA expression was examined by western blot analysis or RT-qPCR. The results are shown as the means ± SD of triplicate experiments. **P<0.01. ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

and either DMSO, 0.5 μM gefitinib, 0.2 μl/ml ECC, or the combination of gefitinib and ECC were added to the embryo culture water, and refreshed every 24 h for 5 days (Fig. 6A). The anticancer effects of ECC were confirmed in both the HCC827 and HCC827GRKU cells. This result was consistent with the *in vitro* results, in which the HCC827GRKU cells were more sensitive than the HCC827 cells to ECC (Fig. 6B). In addition, the gefitinib-, ECC-, and the gefitinib and ECC combination-treated embryos were compared and found to have significantly fewer cancer cells than the control group; however, no significant differences were observed between the treatment groups (Fig. 6C). Unexpectedly, the treatment of zebrafish with ECC alone or in combination with gefitinib resulted in the significantly increased survival of the zebrafish compared to treatment with gefitinib alone (Fig. 6D). This result indicated that ECC had a more specific toxicity against cancer cells than normal cells, consistent with the *in vitro* results.

Discussion

EGFR-TKIs are some of the most effective therapeutic drugs against NSCLCs with EGFR mutations. However, the various adaptive and acquired resistance mechanisms reported have significantly limited the efficacy of EGFR-TKIs and, consequently, the current chemotherapeutic strategies for NSCLCs. Therefore, there is a need to overcome GR resistance to EGFR-TKIs, as GR resistance in lung cancer results in more aggressive cells with an increased viability, proliferation and metastatic ability.

Apoptosis is the natural process through which the elimination of unwanted or damaged cells that present a threat to the health of an organism occurs. This process is highly controlled, and the Bcl family of proteins, which comprises anti-apoptotic proteins and pro-apoptotic proteins, serves as the main regulator of this process (22,23). The anti-apoptotic

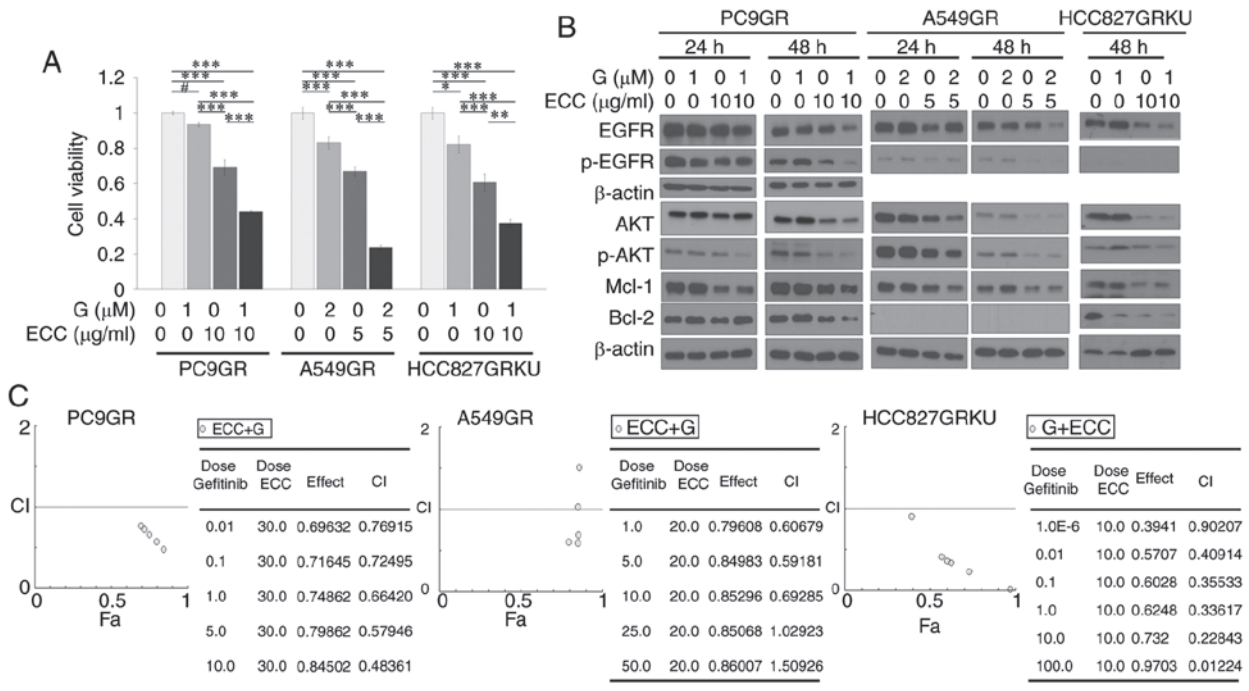

Figure 5. ECC synergistically enhances the activity of gefitinib in GR cells. (A) Cell viability was assessed by MTT assay following treatment of the PC9GR, A549GR and HCC827GRKU cells with the indicated concentrations of of gefitinib alone, ECC alone, or co-treatment with gefitinib and ECC for 72 h. (B) Western blot analysis was performed with the indicated antibodies following treatment with the indicated concentrations of gefitinib, ECC and the combination of gefitinib and ECC for 24 and/or 48 h. (C) The CI of gefitinib and ECC were calculated and the Fa-CI plots generated by the Chou-Talalay method using data obtained from MTT assay following co-treatment with the indicated concentrations of each drug for 72 h. CI values of <1, 1 and >1 indicate synergism, additive effects and antagonism, respectively. The results are shown as the means ± SD of triplicate experiments. *P<0.05, **P<0.01 and ***P<0.001; the hash symbol (#) indicates that there were no significant differences. ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

proteins, Mcl-1 and Bcl-2, play important roles in the maintenance of cell viability and survival, but not proliferation, through the interaction of several other regulators of apoptosis. The overexpression of Mcl-1 and/or Bcl-2 has been shown to facilitate chemoresistance in various types of cancer (24-26), and has been suggested as a therapeutic target. Therefore, efforts have been made to develop drugs targeting Mcl-1 and Bcl-2 to induce chemosensitization and overcome chemoresistance (27-29). Mcl-1 has been suggested to be a critical survival factor in lung cancer as it promotes cancer cell migration ability and epithelial-mesenchymal transition (11,30-32). A previous study demonstrated that the overexpression of Mcl-1 increased the viability of cancer cells following exposure to cytotoxic chemotherapeutic agents and EGFR-TKIs (30).

Conversely, the inhibition of Bcl-2 by various methods (gene suppression and inhibitors) has been shown to increase the sensitivity of lung cancer cells to EGFR-TKIs (26,30,33). This highlights the role of Mcl-1 and Bcl-2 in EGFR-TKI resistance, and indicates that combination treatment comprising EGFR-TKI and Mcl-1 and/or Bcl-2 may exert synergistic effects.

Therefore, the present study evaluated the expression of the anti-apoptotic molecules, Mcl-1, Bcl-2 and Bcl-xL, as well as EGFR signaling molecules, in established GR lung cancer cell and compared it with the expression in their parental cell PC9, A549 and HCC827. The PC9GR cells exhibited a higher expression of EGFR signaling molecules and Bcl-2, but not of Mcl-1. The A549GR cells exhibited a higher expression of AKT/p-AKT and Mcl-1, but not of EGFR/p-EGFR and Bcl-2. The HCC827GRKU cells exhibited a higher expression of AKT/p-AKT, Bcl-2 and Mcl-1. These data suggested that the cellular response to EGFR-TKIs is dependent on the EGFR mutation status, as described in other studies (34,35), which results in a variety of resistance mechanisms to EGFR-TKIs. In the present study, cell viability, migration and invasion assays were thus performed to investigate the physiological anticancer effects of ECC in GR cells. ECC effectively inhibited GR cell viability, migration and invasion. Simultaneously, ECC suppressed EGFR signaling through AKT, regardless of the EGFR mutation status, resulting in the inhibition of GR cell survival. However, ECC exerted minimal toxicity on the normal bronchial cell line, BEAS-2B, unlike the alkaloid extract, berberine; this was confirmed in the *in vivo* model. The dose of ECC in the *in vivo* model was selected, such that it did not pathophysiologically affect survival or induce damage in the zebrafish themselves, but only affected the cancer cells. Therefore, even if the anticancer effect of ECC was not greater than that of gefitinib alone or the combination treatment *in vivo*, the increased survival of zebrafish indicated the specific toxicity of ECC against cancer cells, but not against normal cells, which is crucial for the development of novel anticancer drugs. Cell cycle and TUNEL assays revealed that ECC induced the apoptosis of GR cells through the suppression of Mcl-1, Bcl-2 and Bcl-xl, and the promotion of the expression of cleaved caspase-3 and PARP. As ECC suppressed anti-apoptotic and EGFR/AKT signaling, its synergistic effects with the EGFR-TKI, gefitinib, in GR cells to overcome EGFR-TKI resistance, were evaluated in cells treated with both gefitinib and ECC, and the CI was calculated. The results revealed that ECC treatment re-sensitized the GR cells to gefitinib synergistically through

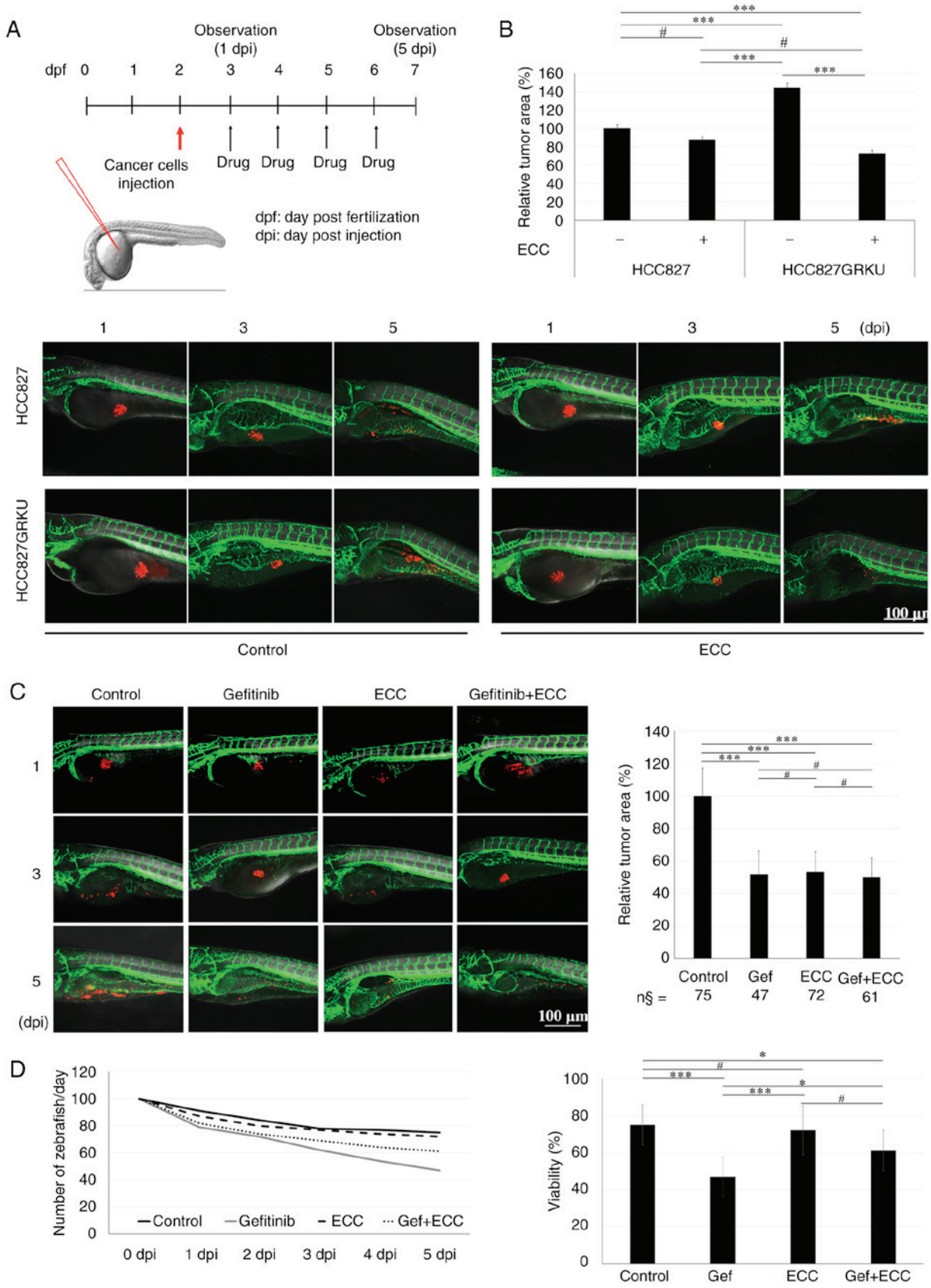

Figure 6. ECC exhibits specific anticancer cell effects *in vivo*. (A) Scheme of the *in vivo* experiment. (B) Representative confocal images of CM-Dil-labelled cancer cells (red) in the vasculature (green) of Tg(FIK1:EGFP) zebrafish larvae, with smaller cancer cell volume following drug treatment compared with the control group (bottom panel). The area penetrated by CM-Dill-labeled cancer cells was quantified (upper panel). (C) Representative confocal images of CM-Dil-labeled cancer cells (red) in the vasculature (green) of Tg(FIK1:EGFP) zebrafish larvae, with smaller cancer cell volume after drug treatment compared with the control group (left panel). The area penetrated by CM-Dil-labelled cancer cells was quantified (right panel). (D) Number of zebrafish with or without treatment of gefitinib, ECC, or combination was counted every day (left panel) and the viability of zebrafish was compared between each group (right panel). *P<0.05 and ***P<0.001; the hash symbol (#) indicates that there were no significant differences. ECC, extract of *Coptis chinensis*; GR, gefitinib-resistant.

the inhibition of EGFR-AKT signaling and the anti-apoptotic proteins, Bcl-2 and Mcl-1.

The fact that in the present study, no tumor xenograft mouse model was used validating the anti-tumor effect of ECC addition to the zebrafish tumor model, which can provide more convincing evidence to the present data and the fact that ECC is a multi-component formulation, rather than a single compound, may be a limitation of this study. However, it should considered that the very weak cytotoxic effects of ECC on normal cells compared to those of berberine, a known alkaloid extracted from ECC, may arise due to the multi-component nature of ECC.

Collectively, the present study found that ECC exerted anticancer effects through the suppression of EGFR/AKT signaling and induced apoptosis via the suppression of the anti-apoptotic proteins, Mcl-1 and Bcl-2, which were overexpressed in GR cells. Moreover, combination treatment with ECC synergistically enhanced GR cell sensitivity to gefitinib, regardless of the EGFR mutation status *in vitro* and increased the viability of normal cells and survival of zebrafish *in vivo*. These results indicated the potential role of ECC in the treatment of EGFR-TKI-resistant NSCLCs, particularly in combination with EGFR-TKI therapy, with minimal side-effects.

Acknowledgements

The abstract was presented and published as a poster (no. P-35-049) in supplement (vol. 9, issue S1): The 44th FEBS Congress July 6-11, 2010 in Krakow, Poland.

Funding

The present study was supported by the Bio-Synergy Research Project (NRF-2014M3A9C4066487) of the Ministry of Science, ICT and Future Planning through the National Research Foundation and by the Basic Science Research Program Grants (NRF-2017R1A2B4003233 and NRF-2019R1A2C1083909) from the National Research Foundation of Korea, which is funded by the Ministry of Education, Science and Technology, Republic of Korea.

Availability of data and materials

All data generated or analyzed during the study are included in this published article or are available from the corresponding author upon reasonable request.

Authors' contributions

JYL conceived and designed the experiments; JHK, ESK, DK, SHP, EJK, JR, WMY, IJH, HS and IJH conducted the experiments; JHK, ESK, DK, SP, EJK, JR, MJK, WMY, IJH, MJP, WMY and JYL analyzed and interpreted the results. All authors reviewed the manuscript and all authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation of Sookmyung Women's University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
2. Fernald K and Kurokawa M: Evading apoptosis in cancer. Trends Cell Biol 23: 620-633, 2013.
3. Lin Y, Wang X and Jin H: EGFR-TKI resistance in NSCLC patients: Mechanisms and strategies. Am J Cancer Res 4: 411-435, 2014.
4. Huang L and Fu L: Mechanisms of resistance to EGFR tyrosine kinase inhibitors. Acta Pharm Sin B 5: 390-401, 2015.
5. Ng KP, Hillmer AM, Chuah CT, Juan WC, Ko TK, Teo AS, Ariyaratne PN, Takahashi N, Sawada K, Fei Y, *et al*: A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. Nat Med 18: 521-528, 2012.
6. Zhao M, Zhang Y, Cai W, Li J, Zhou F, Cheng N, Ren R, Zhao C, Li X, Ren S, *et al*: The Bim deletion polymorphism clinical profile and its relation with tyrosine kinase inhibitor resistance in Chinese patients with non-small cell lung cancer. Cancer 120: 2299-2307, 2014.
7. Wu SG, Liu YN, Yu CJ, Yang PC and Shih JY: Association of BIM deletion polymorphism with intrinsic resistance to EGFR tyrosine kinase inhibitors in patients with lung adenocarcinoma. JAMA Oncol 2: 826-828, 2016.
8. Costa DB, Halmos B, Kumar A, Schumer ST, Huberman MS, Boggon TJ, Tenen DG and Kobayashi S: BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. PLoS Med 4: 1669-1680, 2007.
9. Pandey MK, Prasad S, Tyagi AK, Deb L, Huang J, Karelia DN, Amin SG and Aggarwal BB: Targeting cell survival proteins for cancer cell death. Pharmaceuticals (Basel) 9: pii: E11, 2016.
10. Delbridge AR and Strasser A: The BCL-2 protein family, BH3-mimetics and cancer therapy. Cell Death Differ 22: 1071-1080, 2015.
11. Yang TM, Barbone D, Fennell DA and Broaddus VC: Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. Am J Respir Cell Mol Biol 41: 14-23, 2009.
12. Martin B, Paesmans M, Berghmans T, Branle F, Ghisdal L, Mascaux C, Meert AP, Steels E, Vallot F, Verdebout JM, *et al*: Role of Bcl-2 as a prognostic factor for survival in lung cancer: A systematic review of the literature with meta-analysis. Br J Cancer 89: 55-64, 2003.
13. Tang F, Mei W, Tian D and Huang D: An evidence-based perspective of *Coptis chinensis* (Chinese Goldthread) for cancer patients. In: Evidence-based anticancer materia medica. Cho WCS (ed.) Springer, NY, pp111-130, 2011.
14. Li J, Ni L, Li B, Wang M, Ding Z, Xiong C and Lu X: *Coptis chinensis* affects the function of glioma cells through the down-regulation of phosphorylation of STAT by reducing HDAC3. BMC Complement Altern Med 17: 524, 2017
15. Auyeung K and Ko J: *Coptis chinensis* inhibits hepatocellular carcinoma cell growth through nonsteroidal anti-inflammatory drug-activated gene activation. Int J Mol Med 24: 571-577, 2009
16. Lulu N, Jiangan L, Weixing Z, Zhiyi Z, Hui L, Jianhui T, Haizhou L and Hongli R: *Coptis chinensis* inhibits growth and metastasis and induces cell apoptosis in non-small cell lung cancer cells. Int J Clin Exp Med 10: 16037-16048, 2017.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Park SH, Kim JH, Ko E, Kim JY, Park MJ, Kim MJ, Seo H, Li S and Lee JY: Resistance to gefitinib and cross-resistance to irreversible EGFR-TKIs mediated by disruption of the Keap1-Nrf2 pathway in human lung cancer cells. FASEB J 32: 5862-5873, 2018.
19. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na II, Yang SH, Kim CH and Lee JC: Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. Lung Cancer 63: 219-226, 2009.

20. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na II, Yang SH, Lee SS, Kim CH, Yoo YD and Lee JC: The role of MET activation in determining the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. Mol Cancer Res 7: 1736-1743, 2009.
21. Chou TC and Martin N: CompuSyn software for drug combinations and for general dose-effect analysis, and user's guide. ComboSyn, Inc. Paramus, NJ, 2007.
22. Czabotar PE, Lessene G, Strasser A and Adams JM: Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nat Rev Mol Cell Biol 15: 49-63, 2014.
23. Wertz IE, Kusam S, Lam C, Okamoto T, Sandoval W, Anderson DJ, Helgason E, Ernst JA, Eby M, Liu J, *et al*: Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. Nature 471: 110-114, 2011.
24. Wei G, Margolin AA, Haery L, Brown E, Cucolo L, Julian B, Shehata S, Kung AL, Beroukhim R and Golub TR: Chemical genomics identifies small-molecule MCL1 repressors and BCL-xL as a predictor of MCL1 dependency. Cancer Cell 21: 547-562, 2012.
25. Karnak D and Xu L: Chemosensitization of prostate cancer by modulating Bcl-2 family proteins. Curr Drug Targets 11: 699-707, 2010.
26. Wu DW, Chen CY, Chu CL and Lee H: Paxillin confers resistance to tyrosine kinase inhibitors in EGFR-mutant lung cancers via modulating BIM and Mcl-1 protein stability. Oncogene 35: 621-630, 2016.
27. Belmar J and Fesik SW: Small molecule Mcl-1 inhibitors for the treatment of cancer. Pharmacol Ther 145: 76-84, 2015.
28. Brumatti G and Ekert PG: Seeking a MCL-1 inhibitor. Cell Death Differ 20: 1440-1441, 2013.
29. Pan R, Ruvolo VR, Wei J, Konopleva M, Reed JC, Pellecchia M, Andreeff M and Ruvolo PP: Inhibition of Mcl-1 with the pan-Bcl-2 family inhibitor (-)BI97D6 overcomes ABT-737 resistance in acute myeloid leukemia. Blood 126: 363-372, 2015.
30. Song L, Coppola D, Livingston S, Cress D and Haura EB: Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol Ther 4: 267-276, 2005.
31. Toge M, Yokoyama S, Kato S, Sakurai H, Senda K, Doki Y, Hayakawa Y, Yoshimura N and Saiki I: Critical contribution of MCL-1 in EMT-associated chemo-resistance in A549 non-small cell lung cancer. Int J Oncol 46: 1844-1848, 2015.
32. Zhang H, Guttikonda S, Roberts L, Uziel T, Semizarov D, Elmore SW, Leverson JD and Lam LT: Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. Oncogene 30: 1963-1968, 2011.
33. Zhang J, Wang S, Wang L, Wang R, Chen S, Pan B, Sun Y and Chen H: Prognostic value of Bcl-2 expression in patients with non-small-cell lung cancer: A meta-analysis and systemic review. Onco Targets Ther 8: 3361-3369, 2015.
34. Lohinai Z, Hoda MA, Fabian K, Ostoros G, Raso E, Barbai T, Timar J, Kovalszky I, Cserepes M, Rozsas A, *et al*: Distinct epidemiology and clinical consequence of classic versus rare EGFR mutations in lung adenocarcinoma. J Thorac Oncol 10: 738-746, 2015.
35. Li K, Yang M, Liang N and Li S: Determining EGFR-TKI sensitivity of G719X and other uncommon EGFR mutations in non-small cell lung cancer: Perplexity and solution (Review). Oncol Rep 37: 1347-1358, 2017.