Neoplasma 2022; 69(3): 648–656

doi:10.4149/neo_2022_211103N1568

Co-treatment of betulin and gefitinib is effective against EGFR wild-type/ KRAS-mutant non-small cell lung cancer by inducing ferroptosis

Wei-Ya YAN, Jian CAI, Jiang-Nan WANG, Yong-Sheng GONG*, Xue-Bing DING*

Department of Cardiothoracic Surgery, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou, Jiangsu, China

**Correspondence: gongyongshen163@163.com; dingxuebing888@126.com*

Received November 3, 2021 / Accepted March 7, 2022

Clinical trials suggest that non-small-cell lung cancer (NSCLC) patients with KRAS mutations and wild-type EGFR have reduced benefits from gefitinib treatment. Ferroptosis is a new form of cell death that plays an important role in mediating the sensitivity of EGFR-TIKs. Here, we explored the antitumor ability of gefitinib in combination with betulin to overcome drug resistance through ferroptosis in wild-type EGFR/KRAS-mutant NSCLC cells. A549 and H460 cells were treated with gefitinib and betulin, and cell viability, apoptosis, and migration ability were assessed using the CCK-8 assay, flow cytometry, and wound-healing assay, respectively. Several cell death inhibitors were used to study the form of cell death. Ferroptosis-related events were detected by performing reactive oxygen species (ROS) and iron level detection, malondialdehyde (MDA) assay, and glutathione (GSH) assay. EMT-associated proteins and ferroptosis-related proteins were detected by using western blotting. A xenograft model was constructed *in vivo* to investigate the role of the combination treatment of betulin and gefitinib in NSCLC tumor growth. Gefitinib in combination with betulin exhibited antagonistic effects on cellular viability and induced cell apoptosis. It also induced ROS accumulation, lipid peroxidation, and GSH depletion and induced ferroptosis-related gene expression. Moreover, ferroptosis inhibitors, but not inhibitors of other forms of cell death, abrogated the effect of gefitinib in combination with betulin. Moreover, it also inhibited the tumor growth of NSCLC *in vivo*. Our findings suggest that gefitinib in combination with betulin is a novel therapeutic approach to overcome gefitinib resistance in EGFR wild-type/KRAS-mutant NSCLC cells by inducing ferroptosis.

Key words: gefitinib; betulin; KRAS mutation; ferroptosis; non-small-cell lung cancer

Lung cancer is still the main cause of cancer-related death with a high incidence and mortality [1]. Non-small cell lung cancer (NSCLC) is the most common histological subtype of lung cancer, accounting for approximately 80% of all lung cancer cases [2]. Although there has been progress in the development of targeted therapies, the 5-year survival rate of NSCLC is only approximately 15%, partly due to drug resistance [1, 3].

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are widely used as targeted therapy in NSCLC harboring mutations that activate EGFR. EGFR-TKIs can inhibit the Ras-Raf-MEK-ERK signaling pathway by blocking the activation of EGFR. However, EGFR-TKIs cannot inhibit the activation of signaling in the presence of wild-type EGFR or KRAS mutations since Ras is the downstream effector of EGFR [4]. KRAS mutations are mutually exclusive to EGFR mutations, so NSCLC patients with KRAS mutations are primarily resistant to EGFR-TKIs, such as gefitinib [5, 6]. Indeed, KRAS mutations were identified in 20–30% of NSCLC patients, resulting in poor overall survival [7]. Therefore, it is urgent to develop new strategies to treat NSCLC presenting KRAS mutations.

Ferroptosis is a newly discovered form of cell death that is distinct from apoptosis, autophagy, and necrosis at the morphological, biochemical, and genetic levels. It is characterized by an iron-dependent accumulation of lipid peroxides [8]. Generally, ferric ion (Fe^{3+}) is imported into cells and subsequently converted to ferrous ion (Fe^{2+}) in the endosome [9]. Excessive amounts of Fe^{2+} lead to the accumulation of lipid ROS, thereby causing ferroptosis [10]. Recent reports have shown that ferroptosis is implicated in multiple diseases, including liver disease, kidney injury, and cancer [11]. Additionally, multiple solid tumor cells, including NSCLC cells, exhibit an evident resistance to apoptosis [12]. But it was suggested that therapy-resistant cancer cells were more likely to be killed by ferroptosis [13]. For example, β-elemene is a new ferroptosis inducer, and combinative treatment of β-elemene and cetuximab is effective against KRAS-mutant colorectal cancer cells [14]. Inhibition of GPX4, a critical regulator of ferroptosis, overcomes resistance to Lapatinib

by promoting ferroptosis in NSCLC cells [15]. Therefore, the induction of ferroptosis has been proposed as a novel strategy against therapy-resistant cancer cells.

Betulin (BT), a natural pentacyclic lupane-type triterpene, exhibits antitumor activity in multiple cancer types, including breast cancer, colorectal cancer, and lung cancer [16–18]. Betulin could induce cell cycle arrest, autophagy, and apoptosis in cancer cells [17]. However, its role in ferroptosis and its sensitivity to EGFR wild-type/KRAS-mutant lung cancer cells are still unknown. Here, we demonstrated that combinative treatment with betulin and gefitinib induced ferroptosis and decreased the viability of EGFR wild-type/KRAS-mutant NSCLC cells, which may provide a potential therapeutic strategy for NSCLC patients with KRAS mutations.

Materials and methods

Cell lines. The human NSCLC cell lines A549 and H460 (wild-type EGFR and KRAS mutation) were purchased from the American Type Culture Collection (ATCC). These cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37 °C in a 5% CO_2 atmosphere.

Reagents and antibodies. Betulin (#HY-N0083) and gefitinib (#HY-50895) were purchased from MCE (Shanghai, China). 3-methyladenine (#S2767), Z-VAD-FMK (#S7023), necrostain-1 (#S8037), ferrostatin-1 (#S7243), liproxstatin-1 (#S7699), and deferoxamine (#S5742) were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies against E-cadherin (#14472), N-cadherin (#13116), Vimentin (#5741S), GPX4 (#52455), FTH1 (#FTH1), HO-1 (#70081), SLC7A11 (#12691), and GAPDH (#2118S) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell viability assay. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) (LJ621, Dojindo, Japan) according to the manufacturer's instructions. Cells were seeded into 96-well flat-bottom plates at the density of 5,000 cells/well and incubated at 37 °C for 24 h. The cells were then treated with varying concentrations of betulin and gefitinib for 24 h. After that, a microplate reader (BioTek Instruments Inc.) was used to read the absorbance at 450 nm. Finally, the obtained optical density (OD) values were analyzed via GraphPad Prism software.

Apoptosis assay. For flow cytometry experiments, cells were plated into 6-well flat-bottom plates at 2×10^6 cells/well and treated with betulin, gefitinib, or a combination of these for 24 h. After treatment, cells were collected for Annexin V-FITC/PI staining according to the manufacturer's instructions (MultiSciences Biotech Co., Ltd., China).

Wound-healing assay. Cells were plated into 6-well flat-bottom plates at 5×10^5 cells/well overnight, and then the cell layer was scratched with a sterile plastic tip in 0.5% serum medium. Then, the cells were washed with PBS three times and cultured with betulin, gefitinib, or a combination of these for 12 h. The wound width of the cell layer was photographed using a microscope at 40× magnification.

ROS detection. Cells were seeded into 6-well flat-bottom plates and treated with betulin, gefitinib, or a combination of these for 24 h. After treatment, cells were incubated with DCFH-DA for 20 min at 37 °C and washed three times with PBS. Then, the cells were collected and fluorescence measured by a microplate reader (Tecan, Switzerland) at 488 nm excitation and 525 nm emission, according to the manufacturer's instructions (Beyotime, China). The protein concentrations were detected by using a BCA protein assay kit (Beyotime, China).

Malondialdehyde (MDA) assay. MDA was detected using an MDA assay kit according to the manufacturer's instructions (Abcam, ab118970). The protein concentrations were detected using a BCA protein assay kit (Beyotime, China).

GSH assay. GSH was detected using a GSH assay kit according to the manufacturer's instructions (Nanjing Jiancheng, China). The protein concentrations were detected using a BCA protein assay kit (Beyotime, China).

Lipid ROS detection. Cells were seeded into 6-well flat-bottom plates and treated with betulin, gefitinib, or a combination of these for 24 h. After treatment, cells were incubated with C11 BODIPY (Amgicam, China) for 30 min at 37 °C in the dark and washed three times with PBS. Then, the cells were collected, and fluorescence was measured by a flow cytometer according to the manufacturer's instructions.

Iron measurement. Cells were seeded into 12-well flat-bottom plates and treated with betulin, gefitinib, or a combination of these for 24 h. After treatment, cells were incubated with Phen Green SK (Thermo Scientific, Fremont, CA, USA) for 20 min at 37 °C and washed three times with PBS. Then, the cells were imaged by inverted fluorescence microscopy (Olympus, Tokyo, Japan).

Western blot analysis. Cells were harvested and lysed in ice-chilled RIPA buffer and protease inhibitors for 30 min (Thermo Scientific, Fremont, CA, USA). Protein concentration was determined using a BCA protein assay kit (Beyotime, China). Equal amounts of proteins were separated in an SDS–PAGE gel and transfer-embedded onto PVDF membranes (Millipore, Billerica, MA, USA). The blotted membranes were blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies at 4 °C overnight, followed by a 3-times wash using TBST. Subsequently, the membranes were conjugated with the respective secondary antibodies for 1 h, followed by a 3-times wash using TBST. An enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) was used to detect the signals.

Animal models. Animal protocols were approved by the Institutional Animal Care and Use Committee at Soochow University. All animal procedures complied with the ARRIVE guidelines and were in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Nude

mice (5 weeks) were purchased from SLAC Int. (Shanghai, China). A549 cells (6×10^7/ml) were collected and mixed with Matrigel (Corning, USA) at a 1:1 ratio by volume. Then, 100 μl cells were injected subcutaneously into the back region of nude mice to generate tumors with a size of 100 mm^3. Mice were randomly divided into four groups (n=5/group): the control group, betulin group (10 mg/kg), gefitinib group (30 mg/kg), and the combined group. The control group was orally administered vehicle, while the betulin group, gefitinib group, and the combined group were orally administered betulin, gefitinib, and betulin plus gefitinib every other day. The tumor size and mice body weight were measured every other day too, and the volume was calculated according to the formula: tumor size (mm^3) = (length × $width^2$) × 0.5.

Statistical analysis. All data were presented as mean ± SD. Comparisons among groups were performed using one-way ANOVA. All statistical analyses were performed with GraphPad Prism software. A p-value <0.05 was considered statistically significant (*p<0.05; **p<0.01, and ***p<0.001).

Results

Combinative treatment of betulin and gefitinib was effective against EGFR wild-type/KRAS-mutant NSCLC cells. We first measured the effect of betulin and gefitinib on EGFR wild-type/KRAS-mutant NSCLC cells A549 and H460. The results showed that both gefitinib and betulin led to dose-dependent inhibition of EGFR wild-type/KRAS-mutant NSCLC cells (Figures 1A, 1B). To investigate the synergetic effect of betulin and gefitinib, the CCK-8 assay and software Calcusyn were used. A combination index (CI) value greater than 1 indicated antagonism, whereas a CI value lower than 1 indicated synergism. An optimal synergetic effect was obtained when 5 μM gefitinib and 1 μM betulin were co-incubated (Figures 1C, 1D, Table 1). Therefore, A549 and H460 cells were treated with 5 μM gefitinib and 1 μM betulin for 24 h. The combination of gefitinib and betulin markedly decreased cell viability compared to that of controls (Figure 1E).

Combinative treatment of betulin and gefitinib induced cell apoptosis and suppressed the migration of EGFR wild-type/KRAS-mutant NSCLC cells. To confirm whether the treatment of betulin and gefitinib inhibited cell proliferation through cell apoptosis, Annexin-V was measured through flow cytometry. The results showed an increasing number of dead cells after treatment with betulin and gefitinib (Figure 2A). Furthermore, to investigate whether the co-treatment of betulin and gefitinib affected cell migration, a wound-healing assay was performed. As shown in Figure 2B, the combinative treatment of betulin and gefitinib markedly suppressed the migration of A549 and H460 cells. Moreover, western blotting was performed to detect several epithelial-mesenchymal transition (EMT)-associated proteins. The results showed downregulation of mesenchymal markers Vimentin and N-cadherin and upregulation of the epithelial marker E-cadherin in A549 and H460 cells after the co-treatment of betulin and gefitinib (Figure 2C). Collectively, the combination of betulin with gefitinib might induce cell apoptosis and reduce the migration of EGFR wild-type/KRAS mutant NSCLC cells by inhibiting EMT.

Ferroptosis inhibitors could partially block cell death induced by a combined treatment of betulin and gefitinib. The combination of betulin with gefitinib significantly decreased cell viability by ~50% in A549 and H460 cells, whereas the apoptosis rates were ~8%, suggesting a non-apoptotic mode of cell death after treatment with betulin and gefitinib. To further investigate the mechanism of the combinative treatment of betulin and gefitinib, A549 and H460 cells were treated with betulin and gefitinib in the absence or presence of several cell death inhibitors. We found that treatment combined with 3-methyladenine (3-MA, an autophagy inhibitor), Z-VAD-FMK (a pan-caspase inhibitor), or necrostain-1 (a necroptosis inhibitor) did not protect cells from death due to the treatment with betulin and gefitinib. This indicated that there were other forms of cell death regulating the cell death induced by betulin and gefitinib (Figure 3A).

Moreover, we found that several ferroptosis inhibitors, namely, ferrostatin-1 (Fer-1), liproxstatin-1 (Lip-1), and deferoxamine (DFO), could almost completely rescue the viability of A549 and H460 cells exposed to betulin and gefitinib (Figure 3B). Therefore, combinative treatment with betulin and gefitinib induced cell death mainly through ferroptosis.

Ferroptosis contributed to cell death of EGFR wild-type/KRAS-mutant NSCLC cells under the treatment of betulin in combination with gefitinib. Ferroptosis is an iron-dependent form of cell death resulting from the catastrophic accumulation of lipid ROS [19]. To confirm whether ferroptosis contributed to cell death under the treatment of betulin in combination with gefitinib, several ferroptosis events were detected in A549 and H460 cells. As shown in Figures 4A and 4B, we found that ROS generation and MDA, which is an oxidative stress marker, were significantly increased after co-treatment of betulin and gefitinib. In contrast, GSH levels

Table 1. The combination index of gefitinib and betulin in A549 and H460 cells.

A549				H460			
Gefitinib (μM)	Betulin (μM)	Effect	CI	Gefitinib (μM)	Betulin (μM)	Effect	CI
1	1	0.0699	1.119	1	1	0.0598	1.096
1	5	0.451	0.735	1	5	0.351	0.84
5	**1**	**0.491**	**0.335**	**5**	**1**	**0.421**	**0.439**
5	5	0.613	0.569	5	5	0.629	0.504
10	1	0.589	0.4	10	1	0.501	0.592
10	5	0.791	0.366	10	5	0.714	0.504
20	1	0.723	0.436	20	1	0.699	0.577
20	5	0.811	0.458	20	5	0.808	0.51

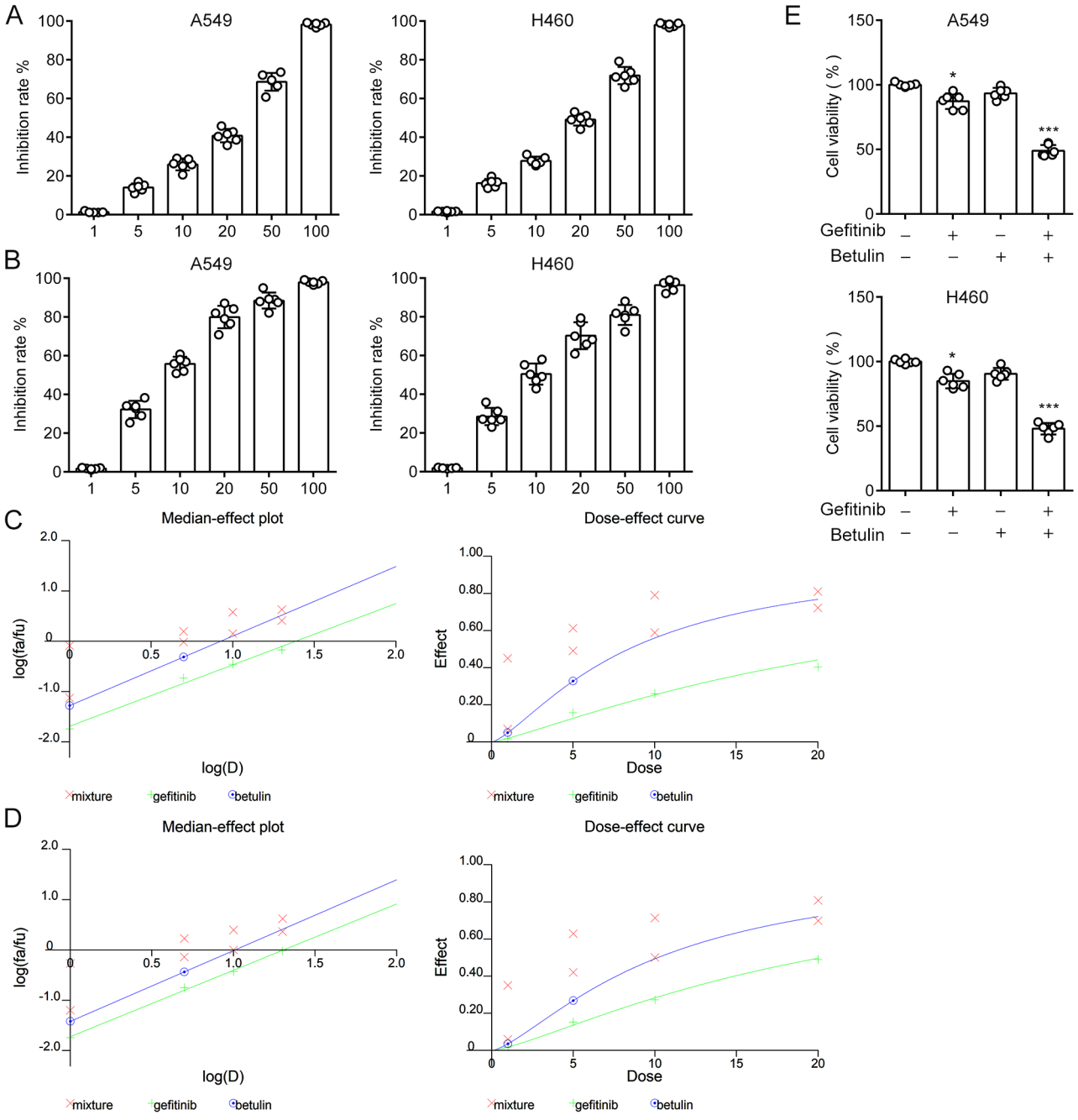

Figure 1. Combinative treatment with betulin and gefitinib was effective against EGFR wild-type/KRAS-mutant NSCLC cells. A549 and H460 cells were treated with gefitinib (A) or betulin (B) (1–100 μM) for 24 h. Cell viability was detected by the CCK-8 assay. Calcusyn software was used to generate a median-effect plot and dose-effect curve of the combination of betulin and gefitinib in A549 (C) and H460 (D) cells. E) A549 and H460 cells were co-treated with betulin (1 μM) and gefitinib (5 μM) for 24 h, and cell viability was detected by the CCK-8 assay. Data from three independent assays were pooled. *p<0.05; **p<0.01; ***p<0.001

were remarkably decreased in cells treated with both betulin and gefitinib (Figure 4C). Moreover, we found that the level of lipid ROS was markedly increased after combinative treatment of gefitinib and betulin (Figure 4D). Accordingly, we determined the intracellular chelatable iron in A549 and H460 cells using the fluorescent indicator Phen Green SK (PGSK), the fluorescence of which is quenched by iron. The proportion of PGSK-positive cells was remarkably decreased under

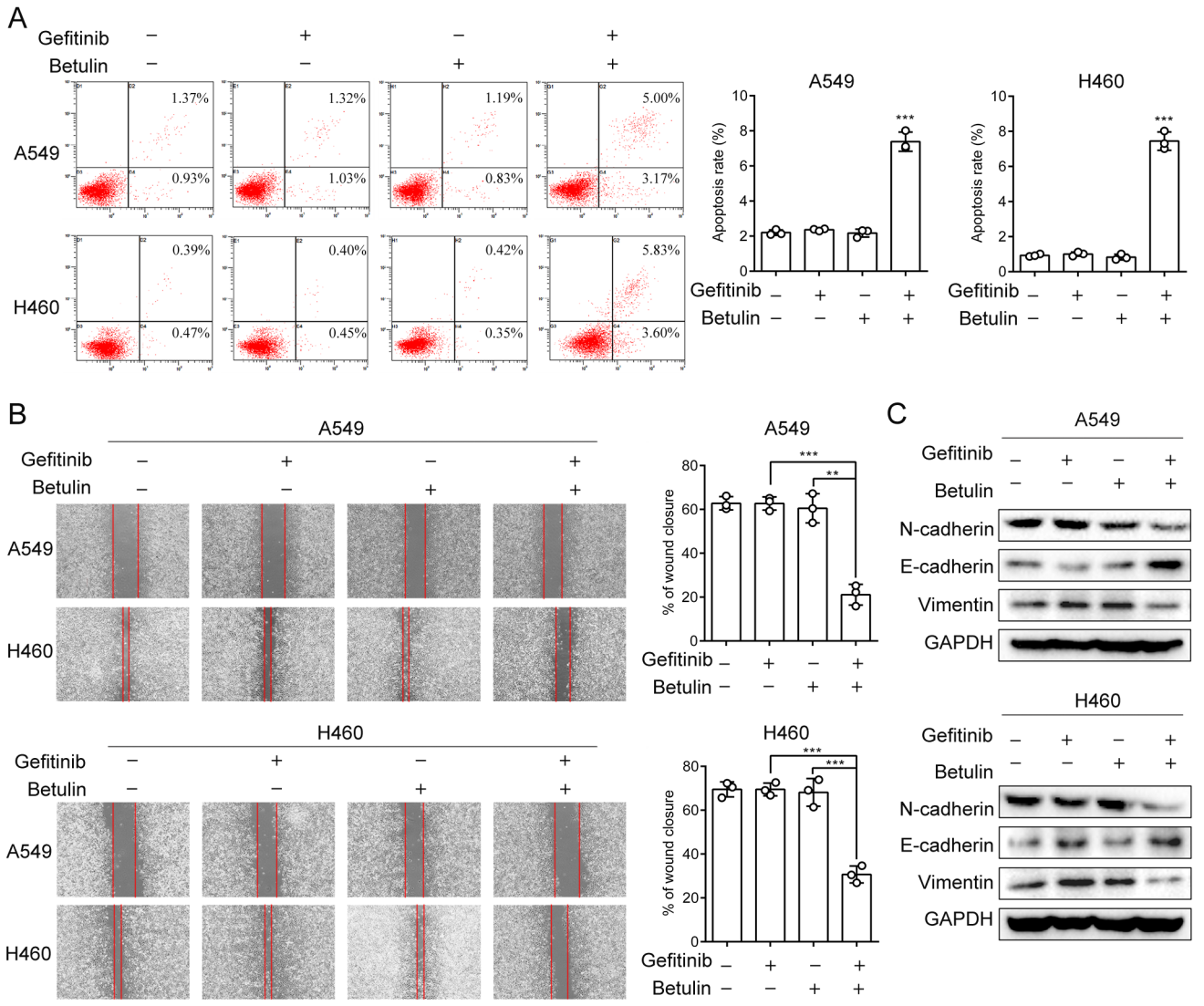

Figure 2. Betulin and gefitinib treatment leads to apoptosis of EGFR wild-type/KRAS-mutant NSCLC cells. A) Representative results of Annexin V-FITC/PI dual staining and quantitative analysis after treatment with betulin (1 μM) and gefitinib (5 μM) for 24 h. Data from three independent assays were pooled. B) Representative results of the wound-healing assay after treatment with betulin (1 μM) and gefitinib (5 μM) for 24 h. C) The expression of EMT-related proteins was detected by western blot after treatment with betulin (1 μM) and gefitinib (5 μM) for 24 h. *p<0.05; **p<0.01; ***p<0.001

betulin treatment in combination with gefitinib (Figure 4E), suggesting that the iron level was increased. Taken together, these results indicated that ferroptosis might play an important role in the cell death of KRAS-mutant NSCLC cells treated with betulin in combination with gefitinib.

To further determine the ferroptosis induced by the combinative treatment of betulin and gefitinib, western blotting was performed to detect the expression of several ferroptosis-related proteins. The expression of SCL7A11, GPX4, and FTH1, which are negative regulators of ferroptosis, was significantly decreased under the combinative treatment of betulin and gefitinib. Moreover, the positive regulatory protein HO-1 was increased (Figure 4F). Taken together, these findings reiterated that the combination of betulin with gefitinib could trigger ferroptosis in KRAS-mutant NSCLC cells.

Combinative treatment with betulin and gefitinib exerted antitumor effects on EGFR wild-type/KRAS-mutant NSCLC cells *in vivo*. To study the therapeutic potential of combinative treatment with betulin and gefitinib, A549 cells were injected into nude mice to generate tumors. The tumor-bearing mice were randomly divided into four groups and orally administered vehicle control, betulin, gefitinib, or betulin plus gefitinib every other day. We found that the

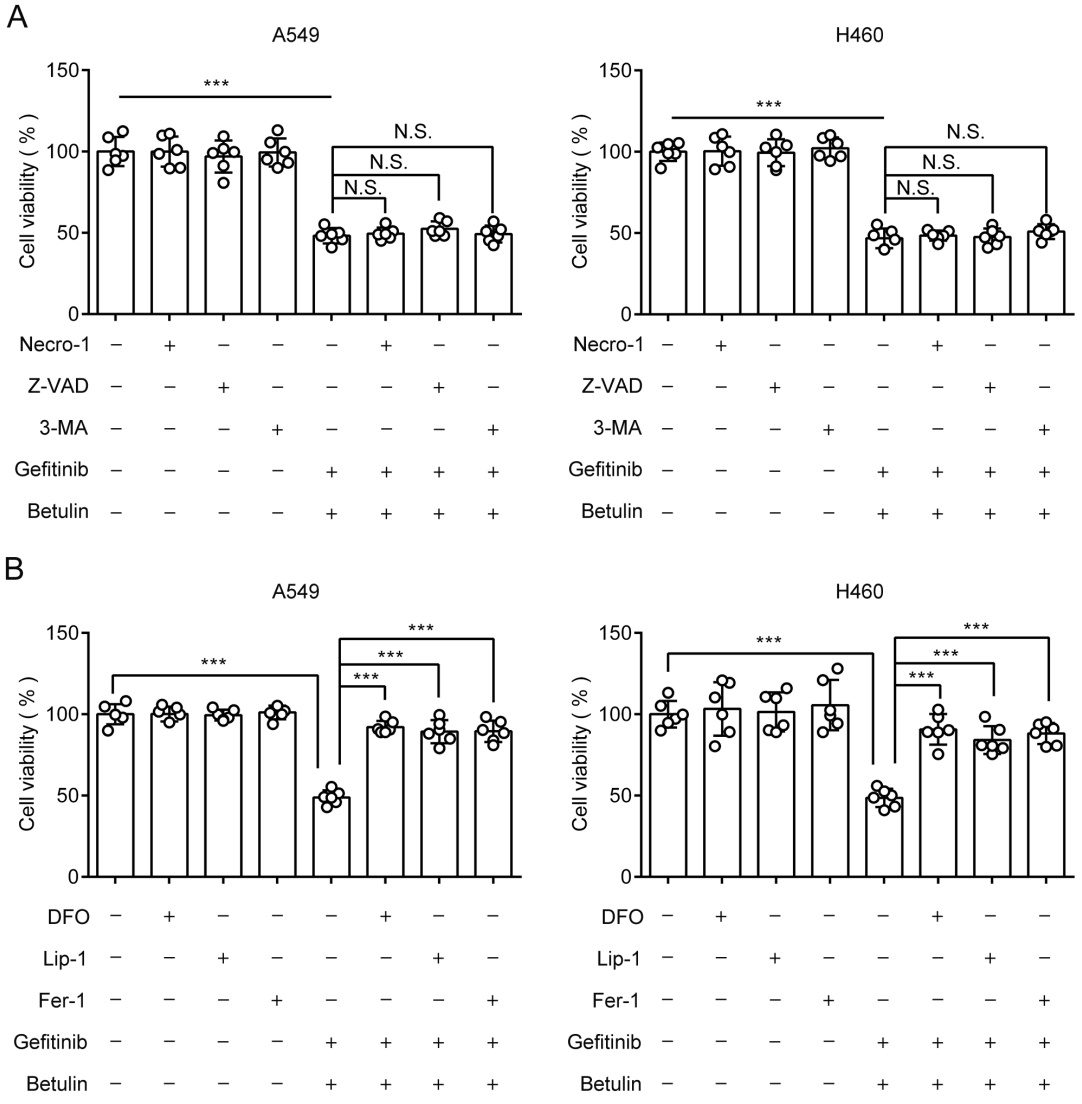

Figure 3. The effects of cell death inhibitors on betulin- and gefitinib-treated EGFR wild-type/KRAS-mutant NSCLC cells. A549 and H460 cells were treated with betulin and gefitinib in combination with other cell death inhibitors (A) or ferroptosis inhibitors (B) for 24 h. Cell viability was detected by the CCK-8 assay. Data from three independent assays were pooled. *p<0.05; **p<0.01; ***p<0.001

combination treatment of betulin and gefitinib significantly inhibited tumor growth (Figure 5A and B). Moreover, the bodyweight of nude mice demonstrated no significant differences among different groups, suggesting desirable safety in combination-treated mice (Figure 5C).

Discussion

Lung cancer is one of the most common malignancies and progressed rapidly [20]. KRAS is one of the most common forms of a mutated oncogene in cancer, and the frequency of KRAS mutations is particularly high in colorectal, pancreatic, and lung cancers [21]. KRAS mutation is often associated with poor prognosis and drug resistance [22]. Gefitinib, an EGFR-targeting agent, has been clinically investigated to treat colorectal cancer, whereas it was shown that gefitinib exhibited no benefit in patients with EGFR wild-type/KRAS-mutant NSCLC [23]. In parallel, betulin was reported to have a wide spectrum of antitumor activity in multiple cancer types, including lung cancer [17]. And betulin in combination with sorafenib, a multi-targeted kinase inhibitor, enhances the anti-tumor effect on NSCLC cells [24]. However, the effects of the combinative treatment of gefitinib and betulin have not been investigated.

In this study, we observed promising effects of the combination of gefitinib and betulin in EGFR wild-type/KRAS-

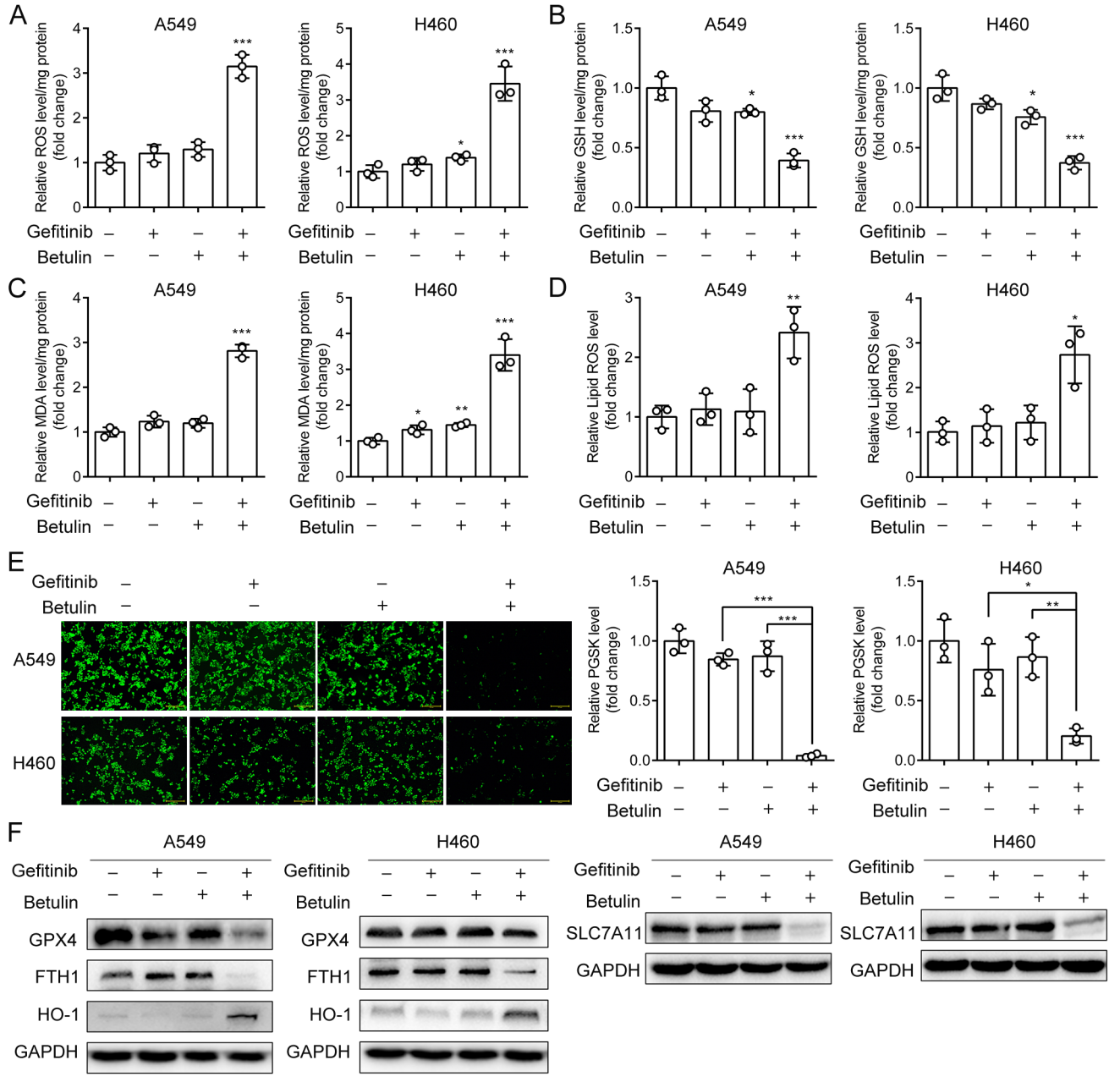

Figure 4. The effects of betulin and gefitinib on ferroptosis-related proteins and events in EGFR wild-type/KRAS-mutant NSCLC cells. A549 and H460 cells were co-treated with betulin (1 μM) and gefitinib (5 μM) for 24 h. Cellular ROS (A), GSH (B), MDA (C), and lipid ROS levels (D) were detected. E) Representative results of PGSK staining and quantitative analysis after treatment with betulin (1 μM) and gefitinib (5 μM) for 24 h. F) The expression of ferroptosis-related genes was detected by western blot after co-treatment with betulin (1 μM) and gefitinib (5 μM) for 24 h. Data from three independent assays were pooled. *p<0.05; **p<0.01; ***p<0.001

mutant NSCLC cells. These *in vitro* studies confirmed that the combination of gefitinib with betulin induced more cell death in EGFR wild-type/KRAS-mutant NSCLC cells A549 and H460 than in cells treated with each compound alone. The optimal synergetic effect was obtained with 5 μM gefitinib and 1 μM betulin. Moreover, gefitinib in combination with betulin obviously regressed EGFR wild-type/KRAS-mutant NSCLC cell migration by inhibiting EMT. More importantly, our results showed that the combinative treatment of gefitinib and betulin markedly suppressed tumor growth *in vivo*.

Ferroptosis is a new form of regulated cell death, which is remarkably distinct from other types and is characterized by the accumulation of ROS from iron metabolism and lipid

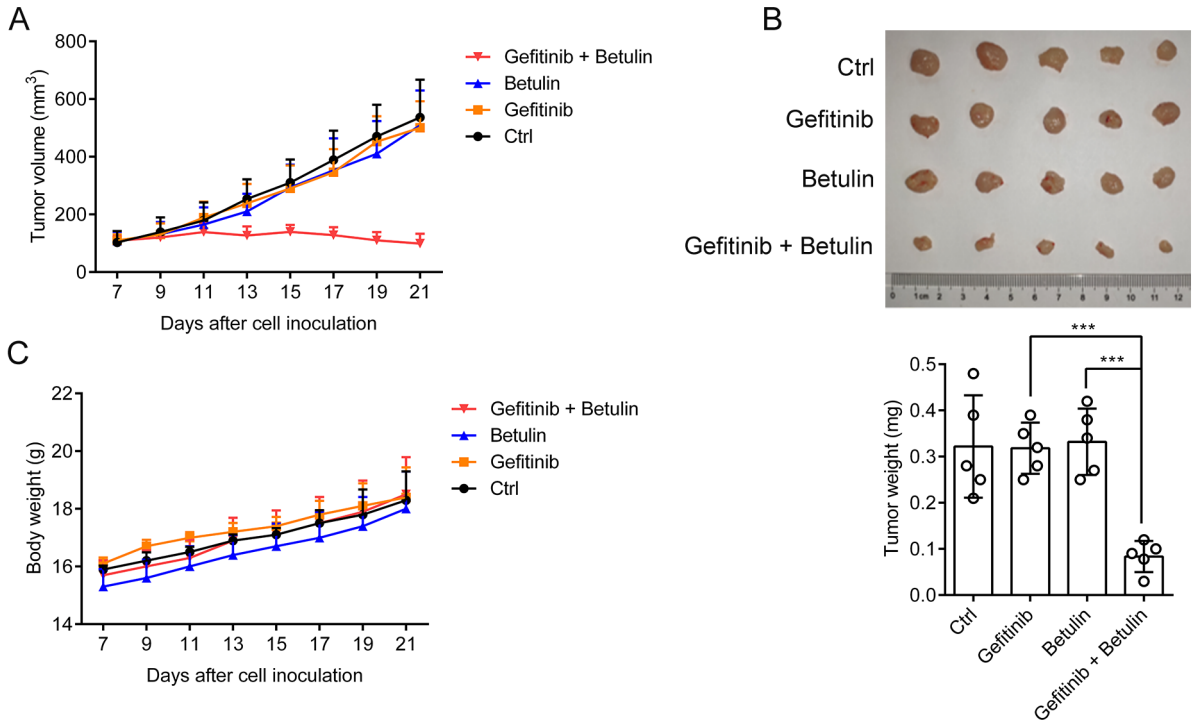

Figure 5. The antitumor effect of combinative treatment with betulin and gefitinib *in vivo*. Nude mice with human A549 subcutaneous xenografts were orally administered vehicle control, betulin (10 mg/kg), gefitinib (30 mg/kg), and betulin plus gefitinib every other day for two weeks. A) Tumor size was monitored every other day using a sliding caliper. The volume was estimated using the following formula: tumor size (mm^3) = (length × $width^2$) × 0.5. After excision from the mice, tumors were photographed and weighed (B). C) The bodyweight of the mice was measured every other day. *p<0.05; **p<0.01; ***p<0.001

peroxidation [10]. There is evidence for the role of ferroptosis in mediating the sensitivity of EGFR-TIKs [14, 25]. Indeed, ferroptosis was reported to mediate the apoptosis of RAS-mutant cancer cells [10]. For example, cetuximab enhances the cytotoxic effect of RSL3 by promoting RSL3-induced ferroptosis in KRAS mutant colorectal cancer cells [26]. Here, we demonstrated that ferroptosis is a key regulator of cell death induced by the combinative treatment of gefitinib and betulin. Our results showed that ferroptosis inhibitors abrogated the cell death induced by the combinative treatment of gefitinib and betulin, whereas autophagy, pan-caspase, or necroptosis inhibitors did not affect cell viability. Several proteins responsible for the regulation of iron metabolism and lipid peroxidation have been involved in ferroptosis. For example, GPX4 and SLC7A11 inhibit ferroptosis by limiting lipid peroxidation, and FHT1 inhibits ferroptosis by reducing the levels of iron whereas HO-1 induces ferroptosis by promoting iron overload [19]. Treatment with gefitinib in combination with betulin induced several ferroptosis events and induced the expression of ferroptosis-related proteins. More importantly, the combinative treatment of betulin and gefitinib remarkably reduced the levels of iron in NSCLC cells, supporting that ferroptosis was induced by betulin and gefitinib treatment.

Taken together, our results suggest for the first time that the combinative treatment of gefitinib and betulin is effective against EGFR wild-type/KRAS-mutant NSCLC cells by inducing ferroptosis. Our study also provides a novel strategy for combinative therapies of NSCLC patients with KRAS mutations.

References

[1] SUNG H, FERLAY J, SIEGEL R, LAVERSANNE M, SOERJOMATARAM I et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021. https://doi.org/10.3322/caac.21660

[2] PARK J, CHO YH, SHIN WJ, LEE SK, LEE J et al. A Ras destabilizer KYA1797K overcomes the resistance of EGFR tyrosine kinase inhibitor in KRAS-mutated non-small cell lung cancer. Sci Rep 2019; 9: 648. https://doi.org/10.1038/s41598-018-37059-8

[3] INDOVINA P, MARCELLI E, MARANTA P, TARRO G. Lung cancer proteomics: recent advances in biomarker discovery. Int J Proteomics 2011; 2011: 726869. https://doi.org/10.1155/2011/726869

[4] PARK J, CHO Y, SHIN W, LEE S, LEE J et al. A Ras destabilizer KYA1797K overcomes the resistance of EGFR tyrosine kinase inhibitor in KRAS-mutated non-small cell lung cancer. Sci Rep 2019; 9: 648. https://doi.org/10.1038/s41598-018-37059-8

[5] TAKEDA M, OKAMOTO I, FUJITA Y, ARAO T, ITO H et al. De novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in EGFR mutation-positive patients with non-small cell lung cancer. J Thorac Oncol 2010; 5: 399–400. https//doi.org/10.1097/JTO.0b013e3181cee47e.

[6] ZHOU B, TANG C, LI J. k-RAS mutation and resistance to epidermal growth factor receptor-tyrosine kinase inhibitor treatment in patients with nonsmall cell lung cancer. J Cancer Res Ther 2017; 13: 699–701. https://doi.org/10.4103/jcrt.JCRT_468_17

[7] CUI W, FRANCHINI F, ALEXANDER M, OFFICER A, WONG H et al. Real world outcomes in KRAS G12C mutation positive non-small cell lung cancer. Lung cancer (Amsterdam, Netherlands) 2020; 146: 310–317. https://doi.org/10.1016/j.lungcan.2020.06.030

[8] GASCHLER M, ANDIA A, LIU H, CSUKA J, HURLOCKER B et al. FINO initiates ferroptosis through GPX4 inactivation and iron oxidation. Nat Chem Biol 2018; 14: 507–515. https://doi.org/10.1038/s41589-018-0031-6

[9] GKOUVATSOS K, PAPANIKOLAOU G, PANTOPOULOS K. Regulation of iron transport and the role of transferrin. Biochim Biophys Acta 2012; 1820: 188–202. https://doi.org/10.1016/j.bbagen.2011.10.013

[10] DIXON SJ, LEMBERG KM, LAMPRECHT MR, SKOUTA R, ZAITSEV EM et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 2012; 149: 1060–1072. https://doi.org/10.1016/j.cell.2012.03.042

[11] STOCKWELL B, JIANG X, GU W. Emerging Mechanisms and Disease Relevance of Ferroptosis. Trends Cell Biol 2020; 30: 478–490. https://doi.org/10.1016/j.tcb.2020.02.009

[12] TISEO M, FRANCIOSI V, GROSSI F, ARDIZZONI A. Adjuvant chemotherapy for non-small cell lung cancer: ready for clinical practice? Eur J Cancer 2006; 42: 8–16. https://doi.org/10.1016/j.ejca.2005.08.031

[13] JIANG X, STOCKWELL B, CONRAD M. Ferroptosis: mechanisms, biology and role in disease. Nat Rev Mol Cell Biol 2021; 22: 266–282. https://doi.org/10.1038/s41580-020-00324-8

[14] CHEN P, LI X, ZHANG R, LIU S, XIANG Y et al. Combinative treatment of β-elemene and cetuximab is sensitive to KRAS mutant colorectal cancer cells by inducing ferroptosis and inhibiting epithelial-mesenchymal transformation. Theranostics 2020; 10: 5107–5119. https://doi.org/10.7150/thno.44705

[15] NI J, CHEN K, ZHANG J, ZHANG X. Inhibition of GPX4 or mTOR overcomes resistance to Lapatinib via promoting ferroptosis in NSCLC cells. Biochem Biophys Res Commun 2021; 567: 154–160. https://doi.org/10.1016/j.bbrc.2021.06.051

[16] JOHN R, DALAL B, SHANKARKUMAR A, DEVARAJAN P. Innovative Betulin Nanosuspension exhibits enhanced anticancer activity in a Triple Negative Breast Cancer Cell Line and Zebrafish angiogenesis model. Int J Pharm 2021: 120511. https://doi.org/10.1016/j.ijpharm.2021.120511

[17] HAN Y, MUN J, JEON H, KEE J, HONG S. Betulin Inhibits Lung Metastasis by Inducing Cell Cycle Arrest, Autophagy, and Apoptosis of Metastatic Colorectal Cancer Cells. Nutrients 2019; 12. https://doi.org/10.3390/nu12010066

[18] KUTKOWSKA J, STRZADALA L, RAPAK A. Hypoxia increases the apoptotic response to Betulinic acid and Betulin in human non-small cell lung cancer cells. Chem-Biol Interact 2021; 333: 109320. https://doi.org/10.1016/j.cbi.2020.109320

[19] TANG D, KROEMER G. Ferroptosis. Curr Biol 2020; 30: R1292–R1297. https://doi.org/10.1016/j.cub.2020.09.068

[20] BARTA JA, POWELL CA, WISNIVESKY JP. Global Epidemiology of Lung Cancer. Ann Glob Health 2019; 85. https://doi.org/10.5334/aogh.2419

[21] FENG H, ZHANG Y, BOS PH, CHAMBERS JM, DUPONT MM et al. K-Ras(G12D) Has a Potential Allosteric Small Molecule Binding Site. Biochemistry 2019; 58: 2542–2554. https://doi.org/10.1021/acs.biochem.8b01300

[22] HAIGIS KM. KRAS Alleles: The Devil Is in the Detail. Trends Cancer 2017; 3: 686–697. https://doi.org/10.1016/j.trecan.2017.08.006

[23] RIELY G, MARKS J, PAO W. KRAS mutations in non-small cell lung cancer. Proc Am Thorac Soc 2009; 6: 201–205. https://doi.org/10.1513/pats.200809-107LC

[24] KUTKOWSKA J, STRZADALA L, RAPAK A. Synergistic activity of sorafenib and Betulinic acid against clonogenic activity of non-small cell lung cancer cells. Cancer Sci 2017; 108: 2265–2272. https://doi.org/10.1111/cas.13386

[25] LORENZATO A, MAGRÌ A, MATAFORA V, AUDRITO V, ARCELLA P et al. Vitamin C Restricts the Emergence of Acquired Resistance to EGFR-Targeted Therapies in Colorectal Cancer. Cancers (Basel) 2020; 12. https://doi.org/10.3390/cancers12030685

[26] YANG J, MO J, DAI J, YE C, CEN W et al. Cetuximab promotes RSL3-induced ferroptosis by suppressing the Nrf2/HO-1 signalling pathway in KRAS mutant colorectal cancer. Cell Death Dis 2021; 12: 1079. https://doi.org/10.1038/s41419-021-04367-3