ORIGINAL ARTICLE

ISAC CYTOMETRY
Journal of Quantitative Cell Science PART A

MIFlowCyt

Yiqi Chutan Tang Reduces Gefitinib-Induced Drug Resistance in Non-Small-Cell Lung Cancer by Targeting Apoptosis and Autophagy

Jue Zhang,[1] Lingling Sun,[1] Jian Cui,[2] Jing Wang,[1] Xiaomin Liu,[3] Thazin Nwe Aung,[2] Zhipeng Qu,[2] Zhuangzhong Chen,[1] David L. Adelson,[2*] Lizhu Lin[1*]

[1]First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, 510000, Guangdong Province, China

[2]Department of Molecular and Biomedical Science, School of Biological Sciences, The University of Adelaide, Adelaide, South Australia, 5005, Australia

[3]Guangzhou University of Chinese Medicine, Guangzhou, 510000, Guangdong Province, China

Received 13 March 2019; Revised 21 May 2019; Accepted 1 July 2019

Grant sponsor: National Natural Science Foundation of China, Grant number81573780

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: *Prof. Lizhu Lin, First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou 510000, Guangdong Province, China. Email: lizhulin05@gmail.com or Prof. David L. Adelson, Department of Genetics and Evolution, School of Biological Sciences, The University of Adelaide, Adelaide, South Australia 5005, Australia. Email: david.adelson@adelaide.edu.au

Published online 14 August 2019 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23869

Abstract

High incidence and mortality rates for non-small-cell lung cancer (NSCLC) lead to low survival rates. Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) are commonly first prescribed for NSCLC patients with *EGFR* mutations. However, most patients with sensitizing *EGFR* mutations become resistant to EGFR-TKI after 9–13 months treatment. Yiqi Chutan Tang (YQCT) has been prescribed as a treatment to this issue for over 20 years. In this report, high-performance liquid chromatography (HPLC) analysis, flow cytometry, western blot analysis, and functional annotation analysis were applied to uncover the molecular mechanisms of YQCT. Our results show the application of YQCT reduces gefitinib-induced drug resistance, induces slight cell cycle arrest, enhances gefitinib-induced apoptosis, and activates the autophagy. These results indicate that at the molecular level YQCT can reduce drug resistance and improve anti-cancer effects when associated with gefitinib, which could be a result of enhancement of apoptosis and autophagy in the EGFR-TKI resistant cells of NSCLC. This research provides a new treatment strategy for patients with EGFR-TKI resistance in NSCLC. © 2019 The Authors. *Cytometry Part A* published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

non-small-cell lung cancer; flow cytometry; molecular mechanism; epidermal growth factor receptor-tyrosine kinase inhibitors; drug resistance

The Global Cancer Observatory 2018 shows that lung cancer has the highest incidence and mortality rates in Asian patients. Non-small-cell lung cancer (NSCLC) is the most common subgroup of lung cancer (1) with platinum-based chemotherapy being the first-line therapy of choice (2), while treatments with epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) have been shown to be more effective with higher survival rates compared to chemotherapy in patients with EGFR-mutated NSCLC (3). However, after 9–13 months of EGFR-TKI therapy, most patients with sensitizing *EGFR* mutations become resistant to EGFR-TKI, which greatly reduces its effectiveness (4). Therefore, there is an urgent need for new strategies to treat NSCLC patients with EGFR-TKI resistance.

One such strategy is the use of Traditional Chinese Medicine (TCM). Its therapy is an important source of effective anti-tumor treatments as well as reducing drug resistance for NSCLC (5). Yiqi Chutan Tang (YQCT) is a TCM formula used to treat NSCLC, which consists of American Ginseng (Xi Yang Shen), Bulbus Fritillariae Thunbergii (Zhe Bei Mu), Radix Ranunculi Ternati (Mao Zhua Cao), Bombyx Batryticatus (Jiang Can), Herba Sarcandrae (Zhong Jie Feng), Indian Iphigenia Bulb (Shan Ci Gu), Rhizoma Pinelliae (Ban Xia), and Lucid Ganoderma (Ling Zhi). It has been shown to inhibit tumor growth in mice (6), reduce drug resistance in lung cancer cells (7,8), prolong the median survival time (9), and reduce chemotherapy-related fatigue in NSCLC patients (10).

Our previous research suggests that YQCT can inhibit EGFR-TKI-resistant tumor growth in vivo, and the mechanism may be related to the regulation of the endoplasmic reticulum stress (ER stress) response by up-regulating CHOP/GADD153 and GADD34 protein expression (11). CHOP is important in ER stress-induced apoptosis, and with CHOP−/− mice have shown to have a low apoptosis rate in response to ER stress (12). Since the PERK/eIF2α/CHOP pathway is essential for inducing autophagy associated with ER stress (13), CHOP also plays an important role in regulating autophagy, with CHOP being able to induce transcription of several autophagy-related genes (ATG). Furthermore, dysregulated autophagy may also trigger ER stress as a feedback mechanism (14). It appears that the regulation of ER stress, apoptosis and autophagy is reflected in more than aspect of YQCT's overall effect. Our previous research has discussed the anti-cancer mechanism of YQCT via ER stress and apoptosis (11,15). In this study, we focus on drug resistance reduction of YQCT in gefitinib-resistant human NSCLC cells H1975 and explore the underlying molecular mechanisms associated with apoptosis and autophagy.

Materials and Methods

Reagents

Dimethylsulfoxide (DMSO) and A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were provided by Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid (BCA) Kit, Radio-Immunoprecipitation Assay (RIPA) lysis buffer, Cell cycle analysis kit, and annexin V-fluorescein isothiocyanate detection kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Cell-light 5-ethynyl-2′-deoxyuridine (EdU) apollo 488 in vitro kit was obtained from Guangzhou Ribo Bio Co., Ltd (Guangzhou, Guangdong, China). Primary antibodies atg3, atg12, β-actin, and secondary antibodies were obtained from Cell Signaling Technology, Inc (Danvers, MA). Western blot detection reagents were provided by Bio-Rad Laboratories (Hercules, CA). Gefitinib was obtained from Selleck Chemicals (Houston, TX).

YQCT Preparation

Herbs were purchased from Guangdong Kang Mei Pharmaceutical Company Ltd (Jieyang, Guangdong, China). Herbs were soaked in double distilled water (1 l) for 30 min, mixed and boiled in eight volumes of water (v/w) for 2 h, and finally re-boiled in eight volumes of water for 1 h. The extracts were filtered and dried by lyophilization. The resulting supernatant was diluted in the cell culture media and filtered with 0.22 μM filter.

High-Performance Liquid Chromatography Analysis

The samples solutions were put into the HPLC (Agilent 1200 HPLC system, Santa Clara, CA) and separated on the chromatographic column C18 (4.6 mm × 250 mm, 2.7 μm). The mobile phase consisted of acetonitrile (A) and 25 mM acetic acid with ammonia (B). The gradient elution program was as follows: 0%–0% A at 0–10 min, 40%–90% A at 35–45 min, 90%–100% A at 45–50 min, 0%–0% A at 51–58 min. The flow rate was 1 ml/min and the detection wavelength was at 254 nm. The injection volume was 10 μl and the column temperature was maintained at 30°C.

Cell Culture

Human *EGFR* mutated lung cancer cells H1975 was obtained by the Chinese Academy of Science (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute−1,640 medium (Thermo Fisher Scientific, MA) with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37°C.

Cell Viability Assay

Briefly, 4×10^3 H1975 cells were seeded in 96-well plates for 24 h and then were incubated with YQCT and gefitinib. After 48 h of incubation, the effects of YQCT and gefitinib on cell viability were detected by the MTT assay. Each well was added with 100 μl of MTT stock solution (0.5 mg/ml) and was incubated for 4 h. Finally, 150 μl of DMSO was added into each well, and the plates were shaken for 10 min for crystal dissolution. Absorbance and cell viability were then determined.

EdU Incorporation and Immunofluorescence Staining

Each well was added with 100 μl of 1:1,000 dilution of EdU-labeling reagent after 48 h cell culture for EdU labeling. The culture medium was discarded after 2 h followed by the addition of 50 μl of cell fixative reagent to each well and incubation at room temperature for 30 min. The medium was discarded and 50 ml (2 mg/ml) glycine was added to each well before further incubation in a shaker for 5 min. About 100 μl of 1 × Apollo reagent was added into the culture medium and incubated in a dark shaker for 30 min. The medium was again discarded and 100 μl of 0.5% TritonX-100 was added. Finally, 100 μl of 1× Hoechst 33342 reagent was added and then incubated in a dark shaker for 30 min. Cells were detected by Leica spectral confocal fluorescent microscopy (Wetzlar, Germany) at 488 nm.

Cell Cycle Distribution Analysis

H1975 cells were seeded in six-well plates overnight. Following adding 12.5 μM of gefitinib combined with or without 0.5 mg/ml YQCT for 48 h incubation. Cells were then re-suspended in 1 ml of phosphate-buffered saline (PBS) containing 1 mg/ml of RNase and 50 μg/ml of propidium iodide (PI), and then were incubated in dark for 30 min at room temperature. A total of 1×10^4 cells were acquired by BD FACSAria™ III Cell Sorter (BD Biosciences, San Diego, CA) at 488 nm to determine cell cycle distribution under the drug treatments. Data were analyzed by FlowJo software (v7; FlowJo LLC, Ashland, OR).

Cell Apoptosis Analysis

Apoptotic cells were quantitated using the annexin V: PI apoptosis detection kit. Cells were treated with 12.5 μM gefitinib combined with or without 0.5 mg/ml YQCT for 48 h, then were trypsinized, washed, and re-suspended at a concentration

of 1×10^6 cells/ml. Then cells were gently re-suspended in 1× binding buffer with 2.5 µl of annexin V: PI in a total volume of 100 µl and incubated in dark for 15 min at room temperature. Apoptotic cells were quantified by BD FACSAria™ III Cell Sorter (BD Biosciences) and data were analyzed by FlowJo software (v7; FlowJo LLC).

Western Blot Analysis

Cell lysate was prepared in RIPA lysis buffer containing 1% phenylmethanesulfonyl fluoride and 1% phosphatase inhibitor. The soluble protein fractions were collected after centrifugation at 1.35×10^4 g for 10 min. Protein concentrations were detected by BCA kit. About 30 mg of proteins were separated on 8–12% dodecyl sulfate, sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE) and were incubated with different antibodies overnight at 4°C. Membranes were incubated with the appropriate secondary antibodies. Signals were measured by an ECL chemiluminescence detection agent.

Data Processing and Functional Annotation

PubChem IDs were submitted into Bioinformatics Analysis Tool for Molecular mechANism (BATMAN) system (16) to identify genes that are regulated by nine major bioactive components in YQCT with a cut-off score of 20. After identifying highly relevant genes, functional enrichment analysis were performed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms using cluster Profiler (v3.5) (17).

Statistical Analysis

SPSS 24.0 software (IBM, NY) was used to performed statistical analysis. Statistical comparisons were performed using Independent-Samples T test or one-way ANOVA test. Data measurement was expressed as mean values ± standard deviation. The value of $P < 0.01$ was considered significantly statistically significant.

Results

Chemical Composition of YQCT

HPLC was used to characterize alcohol extractions of YQCT, which reveals nine major chemical compounds: Rosmarinic Acid, Resorcinol, Isofraxidin, Ganoderic Acid B, Ganoderic Acid C2, Ganoderic Acid A, Peimine, Peiminine, and Beta-Sitosterol (Fig. 1).

YQCT Inhibits Cell Viability, DNA Synthesis and Reduces Gefitinib-Induced Drug Resistance in H1975 Cell Line

The H1975 cell line was used in this research as it has *EGFR* mutations of exon 21 *L858* and exon 20 *T790 M* (18), mutations which are resistant to EGFR-TKI. Gefitinib is a small molecule inhibitor that suppresses *EGFR* tyrosine kinase activity and is the first generation of EGFR-TKI. As shown in Figure 2a, a high dose of gefitinib (50 µM) did not inhibit cell viability in H1975, but it could effectively inhibit cell viability in EGFR-TKI sensitive NSCLC cell line A549. This reveals that the H1975 is more resistant to gefitinib than EGFR-TKI sensitive NSCLC cell line.

EdU is a thymidine analog used to detect cell proliferation. The specific reaction of fluorescent dyes of EdU monitors DNA replication activity and synthesis steps (19,20). In Figure 2b, Hoechst 33342 labeled the nucleus in blue fluorescence, indicating the total number of cells, while the EdU labeled the number of proliferating cells in green fluorescence.

The EdU assay showed that YQCT significantly suppressed the cell proliferation with increasing dose (Fig. 2b), indicating that the application of YQCT inhibited DNA synthesis *in vitro* in a dose-dependent fashion. MTT cell viability assay showed that the use of YQCT significantly inhibited H1975 cell viability in a dose-dependent fashion in 48 h (Fig. 2c). Hence, 0.5 mg/ml of YQCT was used for the study, as this concentration did not show obvious cytotoxicity and could inhibit DNA synthesis as shown in Figure 2b,c.

Compared to the treatment with gefitinib by itself, the treatment with gefitinib combined with YQCT significantly decreased H1975 cell viability in 48 h (Fig. 2d). These results indicate that YQCT may inhibit cell viability, cell proliferation, and DNA synthesis, as well as improving the anti-cancer effect and reducing gefitinib-induced drug resistance in H1975 cells when combined with gefitinib.

YQCT Induces Cell Cycle Arrest, Enhances Gefitinib-Induced Apoptosis while Activating the Autophagy

EdU is incorporated into replicated chromosomal DNA during the S phase of the cell cycle DNA synthesis (19,20). As its

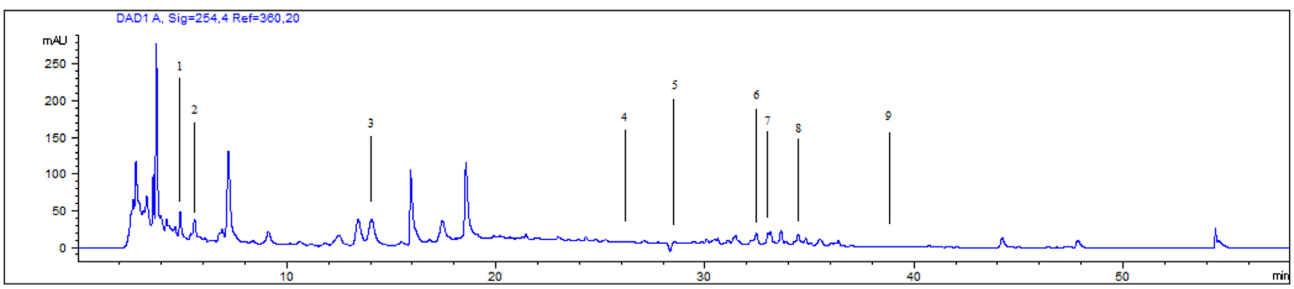

Figure 1. Alcohol extractions of YQCT were qualitatively analyzed with HPLC. The numbers in the chromatograms indicate the constituent peaks 1. Rosmarinic acid, 2. Resorcinol, 3. Isofraxidin, 4. Ganoderic acid B, 5. Ganoderic acid C2, 6. Ganoderic acid a, 7. Peimine, 8. Peiminine and 9. Beta-Sitosterol. A typical chromatogram is shown ($n = 3$). [Color figure can be viewed at wileyonlinelibrary.com]

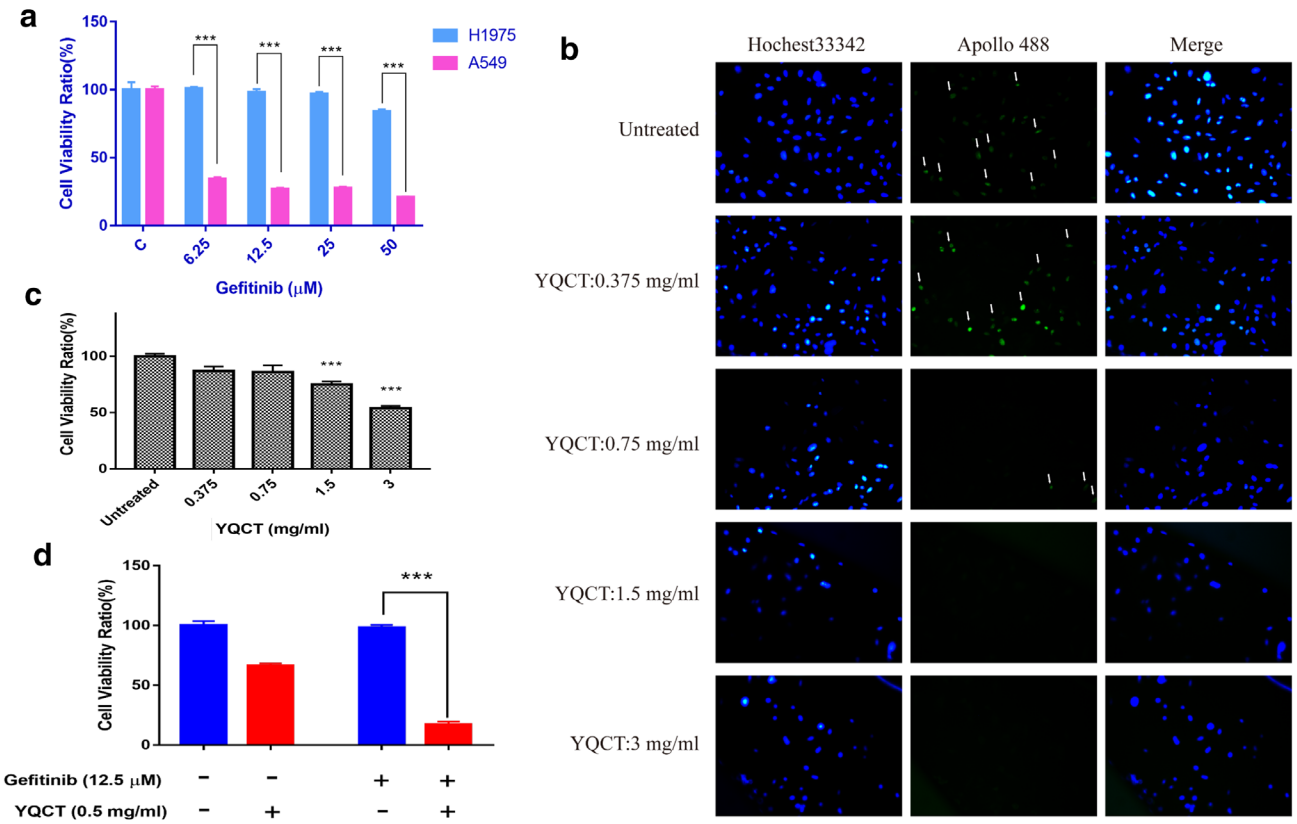

Figure 2. Effects of YQCT on H1975 cells. (**a**) MTT assay for cell viability ratio. H1975 and A549 cells were seeded in 96-well plates (4×10^3 cells/well) overnight and subsequently were treated with 6.25, 12.5, 25, and 50 μM gefitinib for 48 h. (**b**) EdU assay for DNA synthesis. H1975 cells were seeded in 96-well plates (4×10^3 cells/well) overnight and subsequently were treated with 0.375, 0.75, 1.5, and 3 mg/ml YQCT for 48 h, then were stained for EdU and visualized by a fluorescence microscope. (**c**) MTT assay for cell viability ratio. H1975 cells were seeded in 96-well plates (4×10^3 cells/well) overnight and subsequently were treated with 0.375, 0.75, 1.5, and 3 mg/ml YQCT for 48 h. (**d**) MTT assay for cell viability ratio. Cells were seeded in 96-well plates (4×10^3 cells/well) overnight and treated with 12.5 μM gefitinib for 48 h with or without 0.5 mg/ml YQCT. One-way ANOVA test and independent-samples T test were used for analysis, *** indicates $P < 0.01$ ($n \geq 3$). [Color figure can be viewed at wileyonlinelibrary.com]

incorporation is cell cycle dependent, we used flow cytometry to determine whether YQCT can modulate the cell cycle in NSCLC. H1975 cells were treated with 12.5 μM gefitinib either with or without the addition of 0.5 mg/ml YQCT for 48 h. The YQCT combined with gefitinib group (combined group) and YQCT group showed a slight G2/M phase arrest in H1975 cell line compared to the gefitinib group (Fig. 3a,b).

Gefitinib-induced apoptosis in the presence and absence of YQCT was measured in H1975 cells (Fig. 3c,d). Apoptotic cells were detected by flow cytometric analysis. The percentage of late apoptotic cells in the combined group was 1.7 folds higher than gefitinib group in 48 h, indicating YQCT enhanced apoptosis of gefitinib, which revealed that apoptosis might be one of the molecular mechanisms of YQCT for reducing gefitinib-induced drug resistance.

In addition, autophagy occurs upstream of apoptosis and activates it, therefore inducing apoptosis indirectly (21,22). Hence, we examined whether autophagy was also related to apoptosis after YQCT treatment. Atg3 and atg12, key transcription factors for microtubule-associated protein 1A/1B-light chain 3 (LC3) expression, were detected by western blot analysis (Fig. 3e). We observed up-regulated protein expression of atg3 and atg12 in H1975 cells in the combined group compared to gefitinib alone at 48 h, indicating that autophagy of gefitinib was enhanced by YQCT, which revealed that autophagy might be the other molecular mechanism of YQCT for reducing gefitinib-induced drug resistance.

Therefore, it is reasonable to believe that YQCT reduces gefitinib-induced drug resistance in NSCLC by targeting apoptosis and autophagy.

Potential Molecular Mechanisms

To characterize the potential molecular mechanisms of YQCT anti-cancer activity, we submitted the PubChem IDs of the nine principal compounds (Supporting Information Table S1) to the BATMAN (16) database to identify genes that were regulated by these bio-active components. Two hundred and sixty nine genes (gid.csv) were associated with these nine compounds (Supporting Information Table S2), in which 19 genes were relevant to cancer (Fig. 4a and Supporting Information Table S3).

We carried out functional enrichment analysis for these genes based on GO terms and KEGG (Fig. 4b,c and Supporting Information Tables S4 and S5). Significantly over-

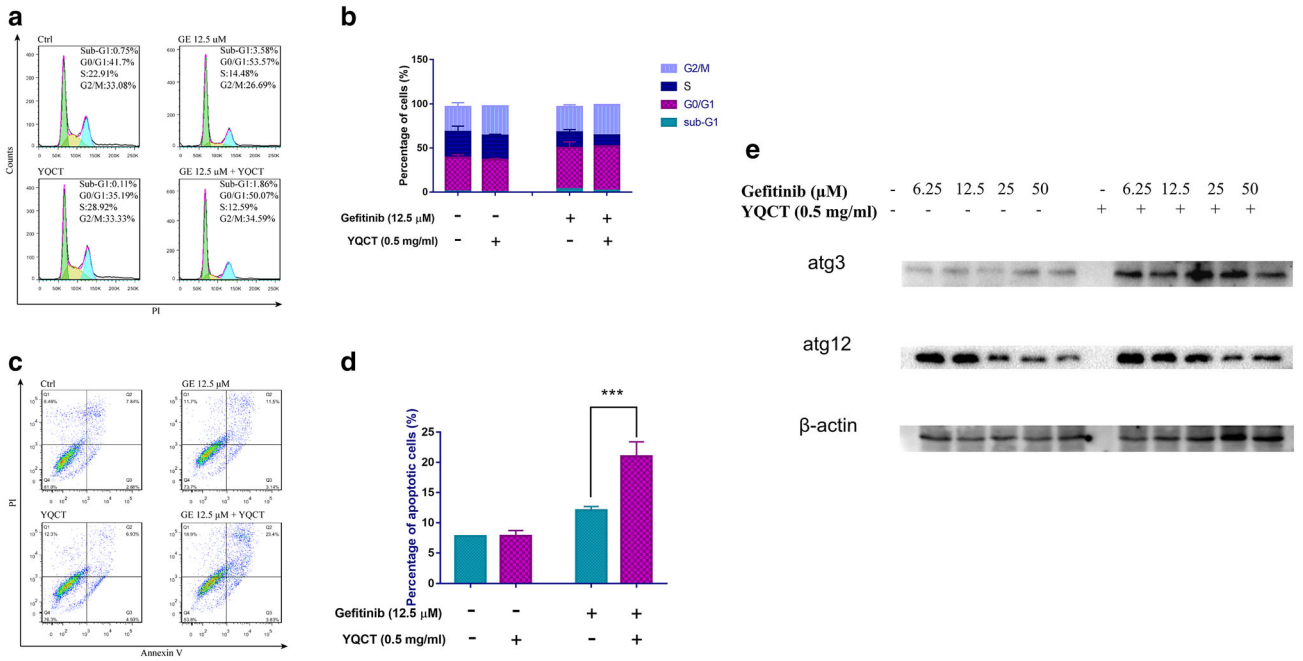

Figure 3. YQCT induces cell cycle arrest, enhances apoptosis and autophagy. (**a**) Effect of YQCT on the cell cycle. Fluorescence-activated cell sorting (FACS) detection of PI stained cells was used to measure the distribution of specific cell populations in Sub-G1, G0/G1, S, and G2/M phases in H1975 cells. Cells were treated with 12.5 μM gefitinib with and without 0.5 mg/ml YQCT for 48 h and then analyzed by flow cytometry. (**b**) Plots the data from (a) to better illustrate the quantitative changes in cell cycle phases. Data were the mean ± SD of independent experiments. (**c**) YQCT induced apoptotic cell death in H1975 cells. Cells were incubated with gefitinib at 12.5 μM in the presence and absence of 0.5 mg/ml YQCT for 48 h, then analyzed by flow cytometry. (d) Plots the data from (c) to better illustrate the quantitative changes in late apoptosis. Data represented the mean ± SD of independent experiments. One-way ANOVA test and independent-samples T test was used for analysis, *** indicates $P < 0.01$. (**e**) Western blot for autophagy proteins. H1975 cells were incubated with gefitinib at 6.25, 12.5, 25, and 50 μM in the presence or absence of 0.5 mg/ml YQCT for 48 h. Atg3, atg12, and β-Actin (dilution ratio: 1:1,000) were visualized via western blot analysis. [Color figure can be viewed at wileyonlinelibrary.com]

represented GO and KEGG functional terms in protein-coding genes were shown in the Supporting Information Tables S6 and S7. We found that apoptosis and cell cycle categories were shown in the GO terms (Fig. 4b). Meanwhile, NSCLC related pathways identified as significantly affected by YQCT were shown in Figure 4c. From the analysis we focused on specific pathways related to cancer, pathways in cancer and pathways in NSCLC were shown in Figure 5a,b.

Discussion

The nine bio-active compounds of YQCT were reported to have anti-cancer effect (23) and reversal of drug resistance (24) by causing cell cycle arrest (25), inducing apoptosis (26) and activating autophagy (27). However, the mechanism of YQCT on drug resistance has not been studied.

EdU is an indicator of cell proliferation (19,20). According to the EdU and MTT assays, 0.5 mg/ml of YQCT can inhibit cell proliferation but shows no obvious cell cytotoxicity. Therefore, its effect of reducing drug resistance appears to be based solely on cell proliferation inhibition. Cell cycle is the basic mechanism of proliferation, differentiation, and cell death. Cell cycle arrest leads to DNA breaks, which ultimately leads to the inhibition of cell proliferation (28). YQCT group and combined group showed arrest in G2/M phase on H1975 cells, which indicates that the YQCT group and combined group could inhibit tumor cell proliferation via G2/M phase arrest, which is also consistent with the result of the EdU assay.

In the treatment of cancer, a promising treatment is manipulating apoptosis by modulation of the key regulators of apoptosis (29). Gefitinib has an effect on apoptosis regulation by competitively binding to the tyrosine kinase domain of *EGFR*, leading to the *EGFR* signaling pathway being blocked. The lack of gefitinib binding to the TKI domain in EGFR-TKI resistant cells leads to a reduction in apoptosis, which reduces the effectiveness of NSCLC treatment (30). Therefore, YQCT combined with gefitinib could be an effective therapy for EGFR-TKI resistance in NSCLC, as it sensitizes the apoptosis induced by gefitinib.

It is reported that loss of autophagy might cause the acquired EGFR-TKI resistance (31). The autophagy-related genes *ATG3* and *ATG12* are involved in tumorigenesis and cancer progression, they also perturb early autophagy pathway, which contributes to autophagosome maturation and formation processes, respectively (32). Therefore, our results indicate YQCT induces autophagy by up-regulating atg3 and atg12, which maybe one of the mechanisms of reducing the acquired EGFR-TKI resistance.

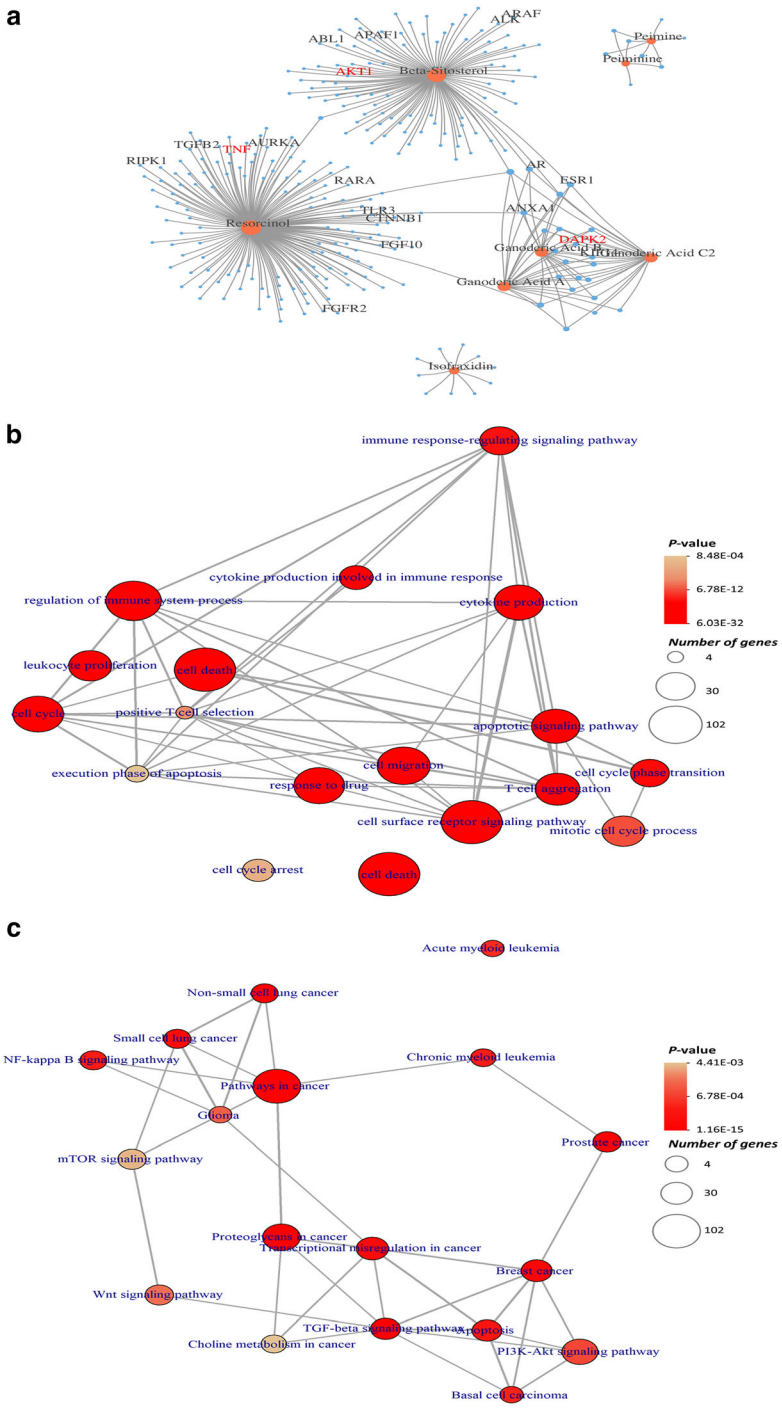

Figure 4. Potential molecular mechanisms of YQCT. (**a**) Genes regulated by YQCT. Orange nodes represented chemical components and blue nodes represented genes. (**b**) GO term enrichment results. The color of each node indicated the significance of the enriched term, the redder meant the higher relevance. The size of each node represented the number of GO term associated genes, the bigger meant the higher association. The names of most significant terms in each cluster were shown. (**c**) KEGG pathway enrichment results. The color of each node indicated the significance of the enriched term, the redder meant the higher relevance. The size of each node represented the number of KEGG term associated genes, the bigger meant the higher association. The names of most significant terms in each cluster were shown. [Color figure can be viewed at wileyonlinelibrary.com]

To find out potential drug resistance reduction mechanisms of YQCT, we find that 269 genes show significant relationship (score > 20) with these nine compounds in YQCT. Three of these genes, *APAF1*, *TNF*, and *DAPK2*, are reported to mediate apoptosis, inhibit cell proliferation and metastasis (33–35). GO terms also show a significant relationship between YQCT and both apoptosis

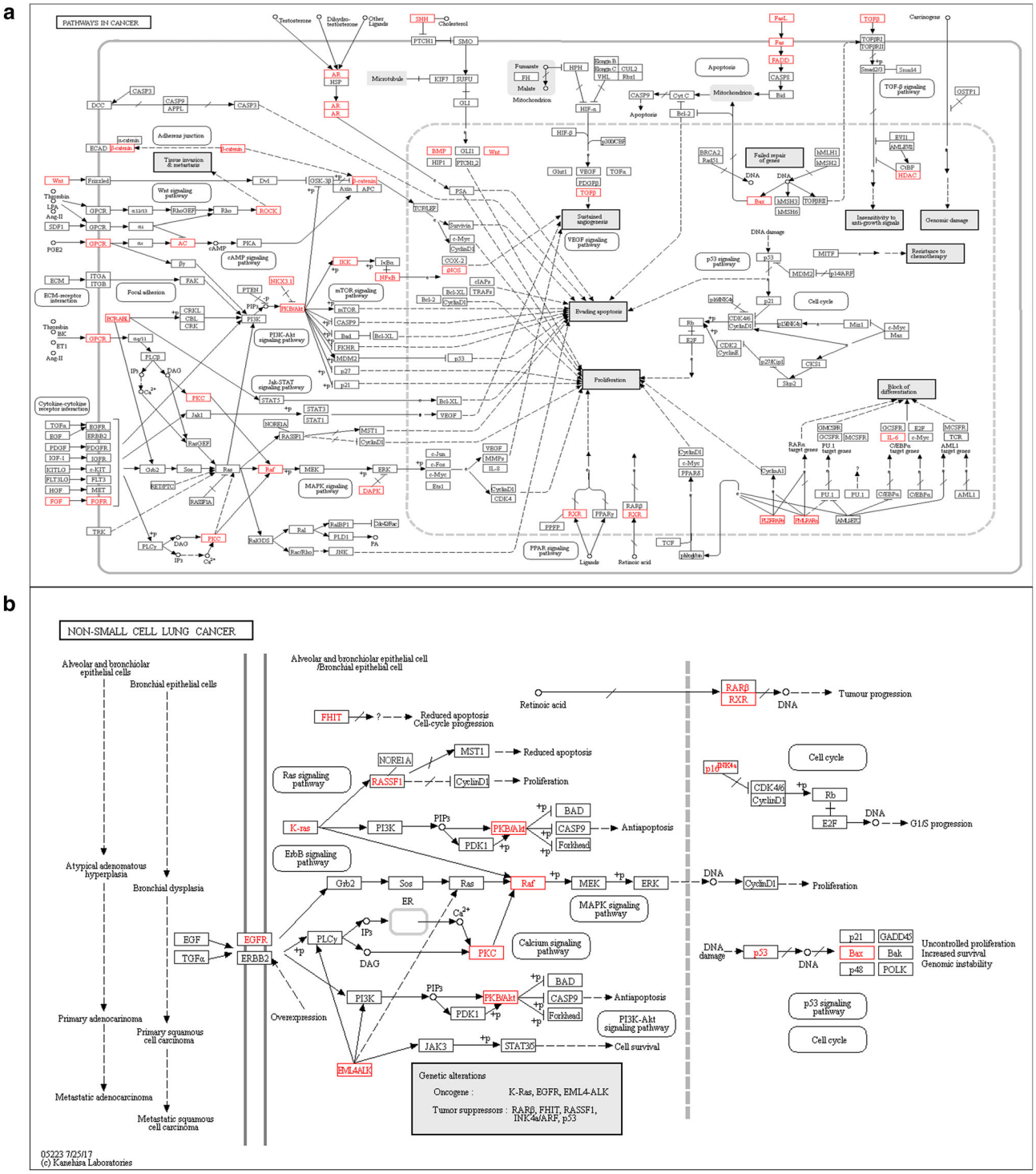

Figure 5. Pathways relevant to YQCT. (**a**) Pathways in cancer. (**b**) Pathways in NSCLC. Potential factors that relevant to YQCT were colored in red. [Color figure can be viewed at wileyonlinelibrary.com]

and cell cycle, as shown in Figure 4b, which is also consistent with the result of the flow cytometry and western blot analysis.

In the pathways in cancer and, specifically NSCLC, the FGF-FGFR and EGF-EGFR pathways are of interest as they are relevant to YQCT. Both of them are upstream effectors of the PI3K-Akt pathway, which relevant to apoptosis. As our previous study indicates that YQCT down-regulates p-akt in A549 NSCLC cells (36), and KEGG enrichment analysis suggests that the PI3K-Akt signaling pathway was affected by YQCT. From this, we speculate that YQCT downregulates the PI3K-Akt

pathway and up-regulates apoptosis by affecting both the FGF-FGFR and EGF-EGFR pathways. These speculation and results provide a potential research direction to screen the relevant pathways in more comprehensive studies.

In summary, we demonstrate YQCT can inhibit cell proliferation, slightly arrest cell cycle at G2/M phase, induce apoptosis, and activate the autophagy. Together these results indicate YQCT has the ability to reduce gefitinib-induced drug resistance, we speculate that this might be through up-regulation of apoptosis and autophagy in EGFR-TKI resistant NSCLC cells.

Asia is the district, which has highest lung cancer new cases in 2018. Although EGFR-TKI are very effective in patients with *EGFR* mutations on lung cancer (37), acquired drug resistance leads to low efficacy and survival rate. Our findings may help in the development of a new strategy for reducing gefitinib resistance during treatment in NSCLC patients with *EGFR* mutations.

ACKNOWLEDGMENTS

We are very grateful to James Galbraith from the University of Adelaide (Australia) and Gee Tang for taking the time to read manuscript in full and offer helpful comments. This work was supported by the National Natural Science Foundation of China (grant number: 81573780).

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

Literature Cited

1. Baltayiannis, N., M. Chandrinos, D. Anagnostopoulos, P. Zarogoulidis, K. Tsakiridis, A. Mpakas, N. Machairiotis, N. Katsikogiannis, I. Kougioumtzi, N. Courcoutsakis, et al., Lung cancer surgery: An up to date. J Thorac Dis 2013;4 (5):S425–S439.
2. Al-Farsi A, Ellis PM. Treatment paradigms for patients with metastatic non-small cell lung cancer, squamous lung cancer: First, second, and third-line. Front Oncol 2014;4:157.
3. Inoue A, Kobayashi K, Maemondo M, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, et al. Updated overall survival results from a randomized phase III trial comparing gefitinib with carboplatin-paclitaxel for chemo-naive non-small cell lung cancer with sensitive EGFR gene mutations (NEJ002). Ann Oncol 2013;24(1):54–59.
4. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, Kris MG, Miller VA, Ladanyi M, Riely GJ. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res 2013;19(8):2240–2247.
5. Jiao L, Dong C, Liu J, Chen Z, Zhang L, Xu J, Shen X, Che J, Yang Y, Huang H, et al. Effects of Chinese medicine as adjunct medication for adjuvant chemotherapy treatments of non-small cell lung cancer patients. Sci Rep 2017;7:46524.
6. Wang S, Lin L, Zhou J, Xiong S, Zhou D. Effects of yiqi chutan tang on the proteome in Lewis lung cancer in mice. Asian Pac J Cancer Prev 2011;12(7):1665–1669.
7. Zhang E, Zhou D, Hou CH. Effects and mechanism of Yiqi Chutan formula on inducing apoptosis of Cisplatin-resistance lung cancer cells by inhibiting Akt Signaling pathway. Traditional Chin Drug Res Clin Pharmacol 2016; 27(3):365–371. (In Chinese).
8. Zhang E, Zhou D, Hou CH. Effects of Yiqi Chutan formula serum on proliferation and cell cycle of cisplatin-resistant lung cancer cells. Shandong Med J 2016; 56(6): 13–15. (In Chinese).
9. Zhou D, Lin L, Tian H, Wu W, Jian Y, Zhang J, Liu K, Deng L, Hu Z, Xu T, et al. Effect on the survival of elderly patients with non - small cell lung cancer treated by the tradiotional Chinese medicine treatment based on Yiqi Huatan method: A multicenter, clinically prospective cohort study. World Chinese Med 2014; 9(7): 833–838. (in Chinese).
10. Guan J, Ouyang M, Xiao Z, Lin L, Zeng K. Effects of Yiqi Chutan decoction on chemotherapy -related fatigue. Liaoning J Trad Chinese Med 2016; 496(6):1211–1213. (in Chinese).
11. Sun L, Fang R, Zhang J, Lin L. Study of Yiqi Chutan recipe in suppressing H1650 lung cancer by regulating endoplasmic reticulum stress response. J Guangzhou Univ Trad Chin Med 2014; 31(4):573–577. (in Chinese).
12. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 2002;109(4):525–532.
13. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. Trends Biochem Sci 2015;40(3):141–148.
14. Deegan S, Saveljeva S, Gorman AM, Samali A. Stress-induced self-cannibalism: On the regulation of autophagy by endoplasmic reticulum stress. Cell Mol Life Sci 2013; 70(14):2425–2441.
15. Li Y, Lin L, Yang J, Fang R, Li J, Zhang J, Yang W. Effects of Yiqi Chutan decoction on the growth of cisolatin-resistant cells of lung cancer and apoptosis of UPR-induced cells. China J Tradit Chin Med Pharm 2014; 29(12):3906–3910. (in Chinese).
16. Liu, Z., F. Guo, Y. Wang, C. Li, X. Zhang, H. Li, L. Diao, J. Gu, W. Wang, D. Li, et al. BATMAN-TCM: A bioinformatics analysis tool for molecular mechANism of traditional Chinese medicine. Sci Rep 2016;6:21146.
17. Yu G, Wang LG, Han Y, He QY. clusterProfiler: An R package for comparing biological themes among gene clusters. Omics 2012;16(5):284–287.
18. Zhao, B.X., J. Wang, B. Song, H. Wei, W.P. Lv, L.M. Tian, M. Li, and S.J.M.M. R. Lv. Establishment and biological characteristics of acquired gefitinib resistance in cell line NCI-H1975/gefinitib-resistant with epidermal growth factor receptor T790M mutation. Mol Med Rep 2015;11(4):2767.
19. Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci U S A 2008;105(7):2415–2420.
20. Zeng C, Pan F, Jones LA, Lim MM, Griffin EA, Sheline YI, Mintun MA, Holtzman DM, Mach RH. Evaluation of 5-ethynyl-2′-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. Brain Res 2010;1319:21–32.
21. Wang, Z., T. Du, X. Dong, Z. Li, G. Wu, and R. Zhang. Autophagy inhibition facilitates erlotinib cytotoxicity in lung cancer cells through modulation of endoplasmic reticulum stress. Int J Oncol 2016;48(6):2558–2566.
22. Lee TG, Jeong EH, Kim SY, Kim HR, Kim CH. The combination of irreversible EGFR TKIs and SAHA induces apoptosis and autophagy-mediated cell death to overcome acquired resistance in EGFR T790M-mutated lung cancer. Int J Cancer 2015;136(11):2717–2729.
23. Sharmila R, Manoharan S. Anti-tumor activity of rosmarinic acid in 7,12-dimethylbenz(a)anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice. Indian J Exp Biol 2012;50(3):187–194.
24. Tang, X.Y., Y.X. Tang, P. Xu, H.Y. Zhou, and L. Han. Effect of Peimine on ERCC1 mRNA and LRP expressions of A549/DDP multidrug resistance cell line. Zhongguo Zhong Xi Yi Jie He Za Zhi 2015;35(12):1490–1494.
25. Sook SH, Lee HJ, Kim JH, Sohn EJ, Jung JH, Kim B, Kim JH, Jeong SJ, Kim SH. Reactive oxygen species-mediated activation of AMP-activated protein kinase and c-Jun N-terminal kinase plays a critical role in beta-sitosterol-induced apoptosis in multiple myeloma U266 cells. Phytother Res 2014;28(3):387–394.
26. Zhao F, Hu Y, Chong C, Lu M, Chen L, Kan W, Chen L, Liu H. Ardisiphenol D, a resorcinol derivative identified from Ardisia brevicaulis, exerts antitumor effect through inducing apoptosis in human non-small-cell lung cancer A549 cells. Pharm Biol 2014;52(7):797–803.
27. Zheng, Z., L. Xu, S. Zhang, W. Li, F. Tou, Q. He, J. Rao, and Q. Shen. Peiminine inhibits colorectal cancer cell proliferation by inducing apoptosis and autophagy and modulating key metabolic pathways. Oncotarget 2017;8(29):47619–47631.
28. Kim H, Lim HY. Novel EGFR-TK inhibitor EKB-569 inhibits hepatocellular carcinoma cell proliferation by AKT and MAPK pathways. J Korean Med Sci 2011;26 (12):1563–1568.
29. Ashkenazi A. Targeting the extrinsic apoptotic pathway in cancer: Lessons learned and future directions. J Clin Invest 2015;125(2):487–489.
30. Grunwald V, Hidalgo M. Developing inhibitors of the epidermal growth factor receptor for cancer treatment. J Natl Cancer Inst 2003;95(12):851–867.
31. Fung C, Chen X, Grandis JR, Duvvuri U. EGFR tyrosine kinase inhibition induces autophagy in cancer cells. Cancer Biol Ther 2012;13(14):1417–1424.
32. Cao QH, Liu F, Yang ZL, Fu XH, Yang ZH, Liu Q, Wang L, Wan XB, Fan XJ. Prognostic value of autophagy related proteins ULK1, Beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer. Am J Transl Res 2016;8(9):3831–3847.
33. Dyari HRE, Rawling T, Chen Y, Sudarmana W, Bourget K, Dwyer JM, Allison SE, Murray M. A novel synthetic analogue of omega-3 17,18-epoxyeicosatetraenoic acid activates TNF receptor-1/ASK1/JNK signaling to promote apoptosis in human breast cancer cells. FASEB J 2017;31(12):5246–5257.
34. Schlegel CR, Fonseca AV, Stöcker S, Georgiou ML, Misterek MB, Munro CE, Carmo CR, Seckl MJ, Costa-Pereira AP. DAPK2 is a novel modulator of TRAIL-induced apoptosis. Cell Death Differ 2014;21(11):1780–1791.
35. Fook-Alves VL, de Oliveira MB, Zanatta DB, Strauss BE, Colleoni GWB. TP53 regulated inhibitor of apoptosis 1 (TRIAP1) stable silencing increases late apoptosis by upregulation of caspase 9 and APAF1 in RPMI8226 multiple myeloma cell line. Biochim Biophys Acta 2016;1862(6):1105–1110.
36. Zhou J, Song N, Sun L, Lin L. Yi-qi-Chu-tan formula induces Anoikis by inhibition of p-AKT in human A549 cancer cell. J Chengdu Univ TCM 2014; 37(2):38–41. (In Chinese).
37. Forde PM, Ettinger DS. Targeted therapy for non-small-cell lung cancer: Past, present and future. Expert Rev Anticancer Ther 2013;13(6):745–758.