

Received: 2018.08.06
Accepted: 2018.09.12
Published: 2018.10.07

Gambogic Acid Induces Apoptosis of Non-Small Cell Lung Cancer (NSCLC) Cells by Suppressing Notch Signaling

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Minghua Zhu**
BCDF 2 **Minjie Wang**
ABEF 3 **Yinfang Jiang**
AD 1 **Hao Wu**
ADF 1 **Guirong Lu**
ACE 1 **Wei Shi**
ADF 1 **Degang Cong**
ABDF 1 **Shaohui Song**
ADE 1 **Keyuan Liu**
ABCDEF 1 **Hao Wang**

1 Department of Cardiothoracic Surgery, The Affiliated Hospital of Hangzhou Normal University, Hangzhou, Zhejiang, P.R. China
2 Department of Respiratory Medicine, Chun'an Second People's Hospital of Hangzhou City, Hangzhou, Zhejiang, P.R. China
3 Department of Cardiovascular Medicine, Hangzhou First People's Hospital, Hangzhou, Zhejiang, P.R. China

Corresponding Author: Minghua Zhu, e-mail: doctor_zhu@zju.edu.cn

Source of support: This study was supported by the Hangzhou Health and Family Planning Commission Key Program (2018Z04: Study on the Mechanism of Inhibiting Radical Development of Non-small Cell Lung Cancer by Traditional Chinese Medicine Active Ingredients Gambogic Acid Induced Programmed Necrosis)

Background: Activation of Notch signaling was found to be associated with cancer. Gambogic acid (GA) was reported to be an anti-cancer agent. This study investigated the anti-cancer effect of GA on human non-small cell lung cancer (NSCLC) cells. Involvement of the Notch pathway was also studied.

Matreial/Methods: GA at 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ was used to incubate A549 and SPC-A1 cells. MTT assay was used to determine the cell viability. TUNEL assay was used to detect the apoptosis. Western blotting was used to evaluate protein expression levels, protein phosphorylation levels, and nuclear translocation levels.

Results: Notch signaling pathway was activated in NSCLC cells. GA treatment significantly inhibited NSCLC cell viability and increased cell apoptosis. GA treatment significantly decreased the expression levels of DLL1, DLL3, DLL4, Jagged1, Jagged2, Bcl2, and PK3K, inhibited NICD nuclear translocation and Akt phosphorylation, and increased expression level of active caspase3.

Conclusions: GA inhibited NSCLC cell viability by inducing apoptosis. Inhibition of the Notch signaling pathway was the mechanism involved in the anti-proliferation effect of GA on NSCLC.

MeSH Keywords: **Apoptosis • Lung Neoplasms • Receptors, Notch**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/912563>



Background

Lung cancer is a common cancer of the respiratory system, and is considered to be one of the most common causes of cancer-related death worldwide [1]. Non-small cell lung cancer (NSCLC) is the major pathological type of lung cancer, accounting for approximately 85% of all lung cancer cases [2]. Although the mortality rate has decreased with the development of surgery and chemotherapy, serious adverse effects and poor prognosis are still of concern. In recent decades, biological extracts from several natural medical herbs have been attracting research attention due to their potent anti-cancer effects [3].

Gambogic acid (GA), also referred as $C_{38}H_{44}O_8$, is one of the bio-active polyprenylated xanthenes extracted from the plant *Garcinia hanburyi*, which has been used in Traditional Chinese Medicine in treatments of inflammatory and parasitic diseases from ancient times [4]. Recently, GA was reported to exert anti-tumor effects against several human cancers such as breast cancer, ovarian cancer, and liver cancer [5–7]. The ability to induce apoptosis was proposed as one of the fundamental mechanisms of the anti-cancer effect of GA. However, the specific molecular mechanisms are still unclear.

Basically, there are 2 major pathways triggering apoptosis. The first one is referred to as the extrinsic or death receptor pathway. The second one is referred to as the intrinsic or mitochondrial pathway, which is regulated by B cell lymphoma/leukemia-2 (Bcl2) and phosphatidylinositol 3-kinase (PI3K) proteins, which are recognized as apoptotic suppressors [8]. Notch signaling was reported to be correlated with the occurrence and development of many human cancers [9]. Notch signaling is activated after Notch receptors bind with their ligands. Then, the Notch intracellular domain (NICD) translocates to the nucleus to initiate transcription of several genes, including Bcl2 and PI3K. Thus, the Notch pathway is believed to be oncogenic [10].

In this study we used 2 human NSCLC cell lines: SPC-A1 and A549. GA was used to incubate these cells. The involvement of Notch/Bcl2 and Notch/PI3K signaling pathways were also investigated. We believe that results from this study will not only add to knowledge of the tumorigenicity of NSCLC, but also provide a theoretical basis for the potential clinical application of GA in patients with NSCLC.

Material and Methods

Cells and treatments

The human NSCLC cell lines A549 and SPC-A1 were provided by the Cell Bank of Typical Preservation Committee, Chinese

Academy of Science. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and an antibiotic mix (Sigma). Cells were cultured in a humidified environment with 5% CO₂ and 95% fresh air at 37°C. Cells were treated with serially diluted GA (Sigma) at concentrations of 0, 0.5, 0.75, and 1.0 μmol/l for 24 h, which was decided according to the results of our pilot study.

Cell viability assessment

The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the protocol described previously [11]. Briefly, A549 and SPC-A1 cells were seeded into the wells of a 48-well plate at cell density of 5×10^3 /well. Cells were then incubated with GA at concentrations of 0, 0.5, 0.75, and 1.0 μmol/l for 24 h and then incubated with 5 mg/ml MTT (Sigma) at 37°C for 4 h. Then, DMSO was added into each well to dissolve the formazan crystals. The absorbance at 490 nm (A490) was detected with a plate reader. The cell viability was then calculated.

Cell apoptosis evaluation

The cell apoptosis of A549 and SPC-A1 cells were assessed by terminal transferase UTP nick-end labeling (TUNEL) assay. Cultured cells were treated with 20 μmol/l proteinase K (Sigma) and fixed with 4% paraformaldehyde. A TUNEL assay kit (Roche) was used to detect the apoptotic cells according to the protocol provided by the manufacturer. The TUNEL-positive cells were tagged with green fluorescence, which was observed with an inverted fluorescence microscope. The images were analyzed using Image J software (NIH).

Western blotting

A cell lysis buffer system (Santa Cruz) was used to prepare the whole-cell extracts on dry ice. The Nuclear Extraction Reagents (Pierce) and Total Protein Extraction kit (Beyotime) were used according to the protocol provided by the manufacturers. The protein concentrations were detected with a BCA protein assay kit (Pierce). Vertical SDS-PAGE was used to separate the protein samples, which were then transferred to the PVDF membranes. After blocking with 10% defatted milk, primary antibodies against Delta-like 1 (DLL1, Sigma-Aldrich, 1: 2000), DLL3 (Sigma-Aldrich, 1: 2000), DLL4 (Sigma-Aldrich, 1: 2000), Jagged1 (Abcam, 1: 2500), Jagged2 (Abcam, 1: 2500), NICD (Cell Signaling Tech, 1: 4000), PI3K (Cell Signaling Tech, 1: 2000), Akt (Cell Signaling Tech, 1: 2000), phosphorylated Akt (p-Akt, Cell Signaling Tech, 1: 2000), Bcl2 (Abcam, 1: 2500), active caspase3 (Abcam, 1: 2000), GAPDH (Abcam, 1: 2000), and Histone H3 (Abcam, 1: 2000) were incubated at 4°C for 8 h. Then, the secondary antibodies were used to incubate the membranes

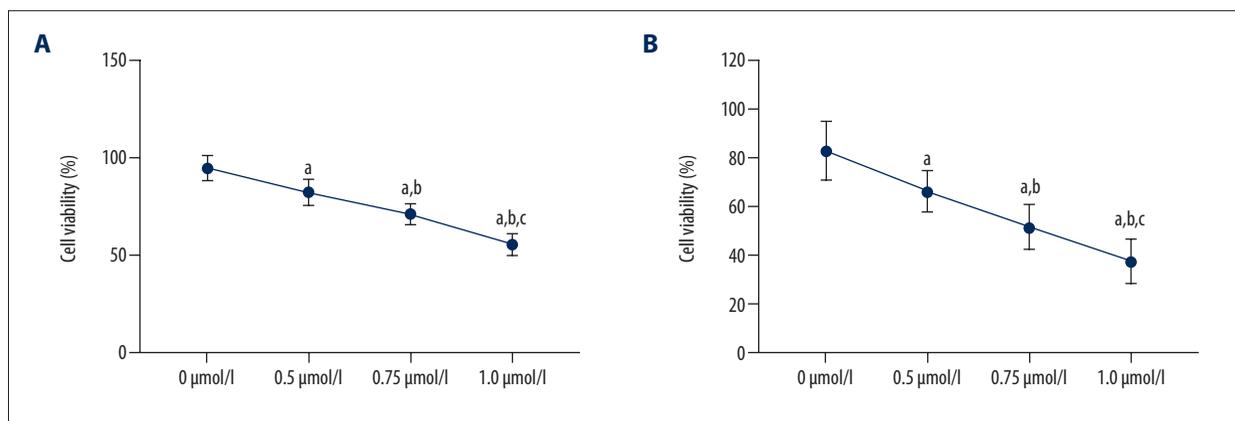


Figure 1. Charts in **A** and **B** indicate the cell viabilities of A549 and SPC-A1 cells were incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 µmol/l for 24 h. [^a Differences were statistically significant with cells incubated with gambogic acid at 0 µmol/l ($P < 0.05$); ^b Differences were statistically significant with cells incubated with gambogic acid at 0.5 µmol/l ($P < 0.05$); ^c Difference was statistically significant with cells incubated with gambogic acid at 0.75 µmol/l ($P < 0.05$)].

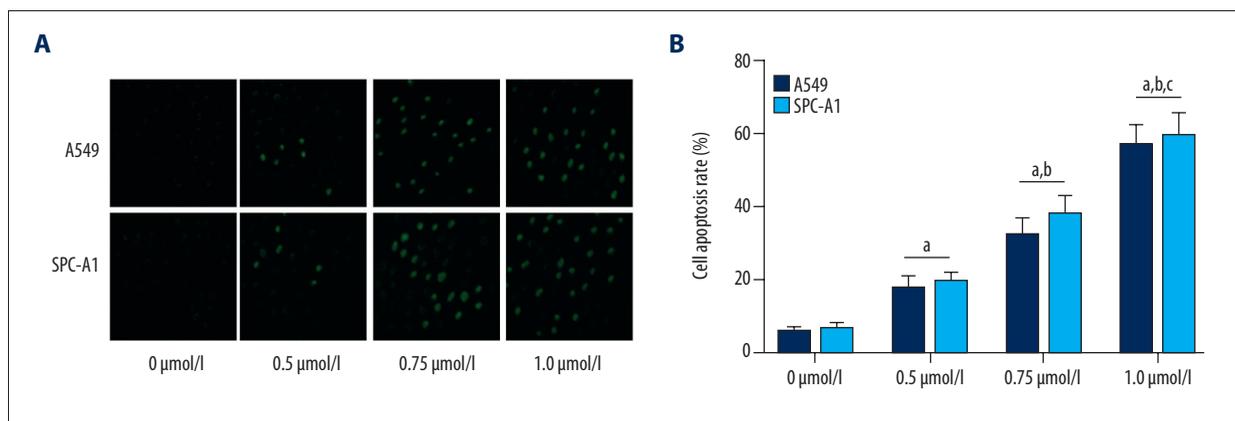


Figure 2. (A) Captured images of TUNEL assay of A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 µmol/l for 24 h. Apoptotic cells were stained with green fluorescence. (B) Columns indicated the apoptotic rate of A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 µmol/l for 24 h. [^a Differences were statistically significant with cells incubated with gambogic acid at 0 µmol/l ($P < 0.05$); ^b Differences were statistically significant with cells incubated with gambogic acid at 0.5 µmol/l ($P < 0.05$); ^c Differences were statistically significant with cells incubated with gambogic acid at 0.75 µmol/l ($P < 0.05$)].

at room temperature for 1 h. An ECL kit (Pierce) was used to develop the membranes, which were then exposed with Gene Genius (Syngene). Image J software was used to analyze the densities of the blots.

Statistical analysis

Data are presented as mean \pm SD and were analyzed using SPSS (version 16.0). Differences between groups were analyzed by *t* test or one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

GA reduced growth of NSCLC cells

The results are demonstrated in Figure 1. GA incubation significantly reduced the cell viability of A549 and SPC-A1 cells in a concentration-dependent manner.

GA induced apoptosis of NSCLC cells

Figure 2 demonstrates the results. After being incubated with GA at various concentrations, apoptosis was detected in A549 and SPC-A1 cells. Moreover, GA induced apoptosis of A549 and SPC-A1 cells in a concentration-dependent manner.

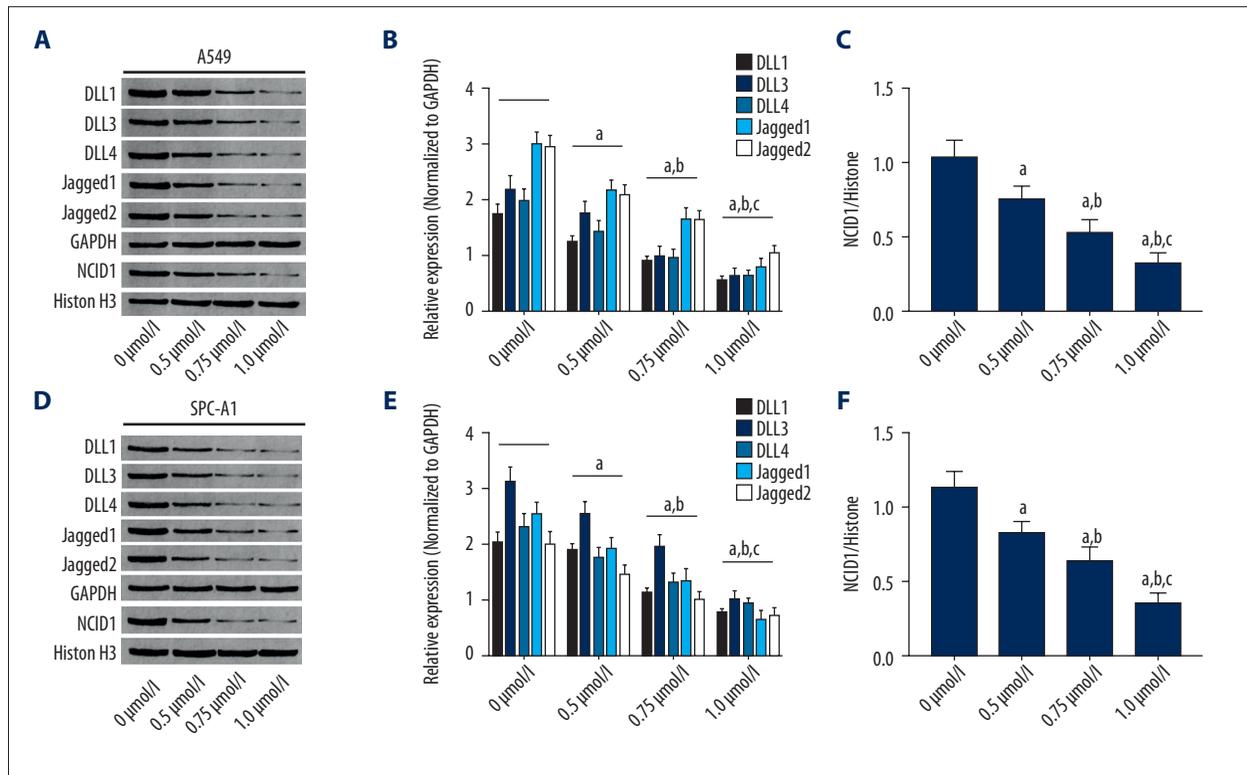


Figure 3. (A, D) Immunoblots of DLL1, DLL3, DLL4, Jagged1, Jagged2, GAPDH, NICD1, and Histone H3 in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. (B, E) Columns indicate the relative expression levels of DLL1, DLL3, DLL4, Jagged1, and Jagged2 (normalized to GAPDH) in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. (C, F) Columns indicate the relative nuclear translocation levels of NICD1 (normalized to Histone H3) in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. [a Differences were statistically significant with cells incubated with gambogic acid at 0 $\mu\text{mol/l}$ ($P < 0.05$); b Differences were statistically significant with cells incubated with gambogic acid at 0.5 $\mu\text{mol/l}$ ($P < 0.05$); c Difference were statistically significant with cells incubated with gambogic acid at 0.75 $\mu\text{mol/l}$ ($P < 0.05$)].

GA inhibited Notch signaling activation in NSCLC cells

The results were demonstrated in Figure 3. The expression levels of DLL1, DLL3, DLL4, Jagged1, and Jagged2, as well as the nuclear translocation of NICD1, increased significantly in A549 and SPC-A1 cells. However, GA incubation dramatically reduced the expression levels of DLL1, DLL3, DLL4, Jagged1, and Jagged2 and nuclear translocation of NICD1 in A549 and SPC-A1 cells in a concentration-dependent manner.

GA suppressed Notch/Bcl2 and Notch/PI3K signaling pathways in NSCLC cells

The results are demonstrated in Figure 4. The phosphorylation level of Akt and the expression levels of PI3K and Bcl2 were increased in A549 and SPC-A1 cells. However, GA administration decreased the phosphorylation level of Akt and the expression levels of Bcl2 and PI3K in a concentration-dependent manner. GA incubation also elevated the expression level of active caspase3 in A549 and SPC-A1 cells in a concentration-dependent manner.

Discussion

Lung cancer is one of the leading malignant cancers worldwide and it was reported that approximately 22 000 cases are diagnosed yearly [12]. The prognosis of lung cancer is relatively poor and the over-all 5-year survival rate is only 16.6% according to previous studies [12]. Like other human malignant cancers, the imbalance of oncogene and anti-oncogene is found during the tumorigenesis, development, and progression of lung cancer. Increased expression levels of several typical oncogenes, such as Bcl2 and PI3K, were reported to be highly correlated with the proliferative ability of many human cancers, including NSCLC [13,14].

After the Notch ligands DLL1, DLL3, DLL4, Jagged1, and Jagged2 bind to Notch receptors, the Notch pathway is activated. The NICD is thus translocated to the nucleus to regulate transcriptions of the target genes, including Bcl2 and PI3K [15]. According to previous studies, the Notch pathway is correlated with occurrence, development, and progression of many human cancers [16].

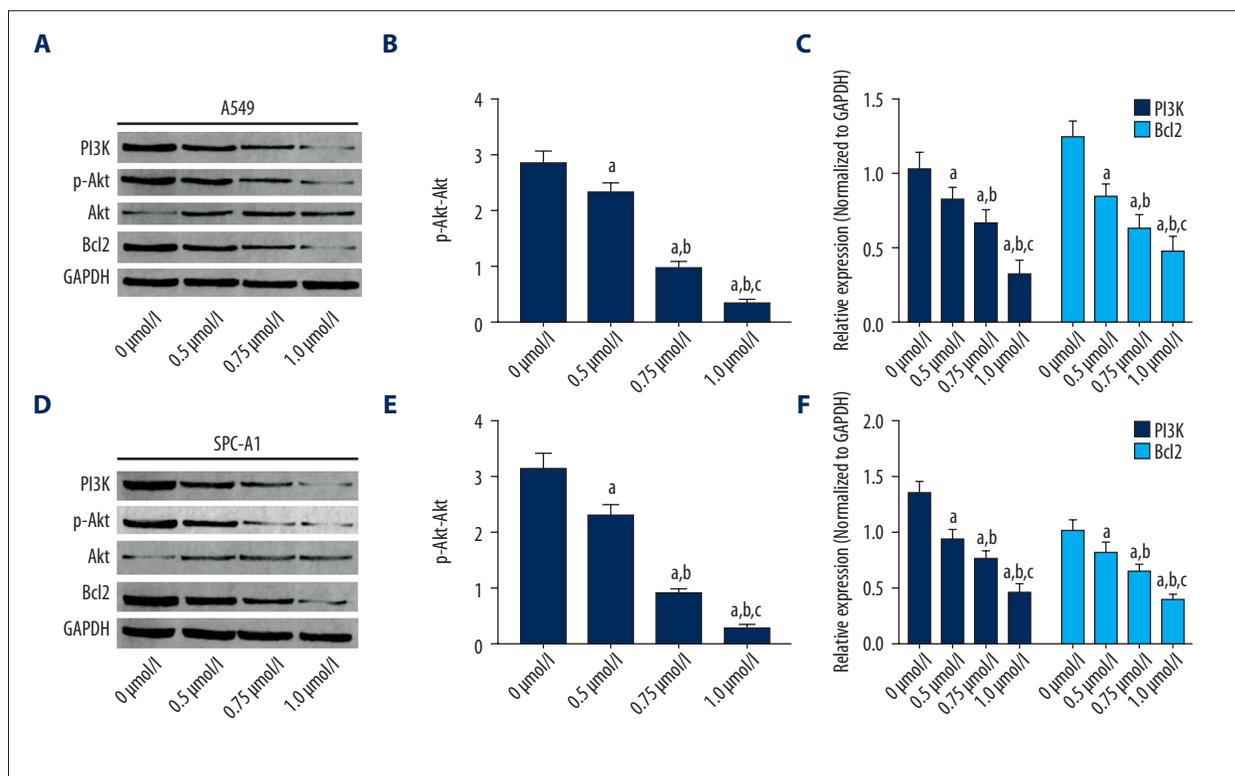


Figure 4. (A, D) Immunoblots of PI3K, p-Akt, Akt, Bcl2, and GAPDH in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. (B, E) Columns indicate the relative phosphorylation levels of Akt (p-Akt/Akt) in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. (C, F) Columns indicate the relative expression levels of PI3K and Bcl2 in A549 and SPC-A1 cells incubated with gambogic acid at concentrations of 0, 0.5, 0.75, and 1.0 $\mu\text{mol/l}$ for 24 h. [° Differences were statistically significant with cells incubated with gambogic acid at 0 $\mu\text{mol/l}$ ($P < 0.05$); ° Differences were statistically significant with cells incubated with gambogic acid at 0.5 $\mu\text{mol/l}$ ($P < 0.05$); ° Differences were statistically significant with cells incubated with gambogic acid at 0.75 $\mu\text{mol/l}$ ($P < 0.05$)].

Abnormal activation of Notch signaling was believed to be associated with malignant phenotypes in several human cancers, including lung cancer [15]. Suppressing Notch signaling was found to result in loss of the malignant phenotype in an experimental animal cancer model and in cultured cancer cells [16]. In this study, we found that Notch signaling was activated in human NSCLC cells, which led to the increased expressions of Bcl2 and PI3K.

The intrinsic apoptotic pathway acts through Bcl2, which is classified as an anti-apoptotic protein. In response to the apoptotic stimuli, the Bcl2 protein binds to the proapoptotic protein bcl-2 homology 123 (BH123), Bax, and Bak, which are located on the outer membrane of mitochondria [17]. Thus, the mitochondrial membrane is stabilized and the formation of permeability transition pores and release of cytochrome c are inhibited [17]. As a result, the apoptosis is suppressed. The PI3K signaling pathway is recognized to be resistant to apoptosis and to promote cancer survival against chemotherapeutic agents [18]. Recruited by receptor tyrosine kinases (RTKs), PI3K

are accumulated on membranes to facilitate the phosphorylation of PIP2 to PIP3, which further recruit serine-threonine kinase Akt to the membrane. Akt is activated by phosphorylation and leads to activation of pro-survival proteins such as I κ B kinase complex (IKK) [19]. In this study, we found that expression levels of the anti-apoptotic proteins Bcl2 and PI3K were increased in human NSCLC cells.

In recent decades, efforts have been made in identifying and developing anti-cancer agents from medical herbs worldwide due to their high effectiveness and low rates of adverse effects. GA is one such agent showing anti-cancer activity [20]. It was reported that GA inhibited cell proliferation, invasion, and migration [21]. GA was reported to inhibit cancer cell proliferation by inducing apoptosis, but the specific molecular mechanisms are still not clear. Moreover, although several investigations mentioned the apoptotic-inducing effect of GA on lung cancer cells, the mechanisms are unclear [22]. In the present study, GA was used to incubate 2 human NSCLC cell lines. The results show that GA reduced NSCLC cell viability by inducing

apoptosis and that GA incubation significantly suppressed activation of the Notch pathway in NSCLC cells. As a result, the expressions of the Notch pathway's target genes, Bcl2 and PI3K, were reduced, triggering apoptosis.

References:

1. Min J, Huang K, Tang H et al: Phloretin induces apoptosis of non-small cell lung carcinoma A549 cells via JNK1/2 and p38 MAPK pathways. *Oncol Rep*, 2015; 34: 2871-79
2. Mirimanoff RO: Neoadjuvant chemoradiotherapy followed by surgery for stage IIIa and IIIb non-small-cell lung cancer (NSCLC): Is it still justified? *Chin Clin Oncol*, 2015; 4: 49
3. Hsu WH, Chang CC, Huang KW et al: Evaluation of the medicinal herb *Graptopetalum paraguayense* as a treatment for liver cancer. *PLoS One*, 2015; 10: e0121298
4. Wang J, Yuan Z: Gambogic acid sensitizes ovarian cancer cells to doxorubicin through ROS-mediated apoptosis. *Cell Biochem Biophys*, 2013; 67: 199-206
5. Zhen YZ, Lin YJ, Li KJ et al: Gambogic acid lysinate induces apoptosis in breast cancer mcf-7 cells by increasing reactive oxygen species. *Evid Based Complement Alternat Med*, 2015; 2015: 842091
6. Tang Q, Lu M, Zhou H et al: Gambogic acid inhibits the growth of ovarian cancer tumors by regulating p65 activity. *Oncol Lett*, 2017; 13: 384-88
7. Lee PN, Ho WS: Antiproliferative activity of gambogic acid isolated from *Garcinia hanburyi* in Hep3B and Huh7 cancer cells. *Oncol Rep*, 2013; 29: 1744-50
8. Fulda S: Modulation of mitochondrial apoptosis by PI3K inhibitors. *Mitochondrion*, 2013; 13: 195-98
9. Yuan X, Wu H, Xu H et al: Notch signaling: An emerging therapeutic target for cancer treatment. *Cancer Lett*, 2015; 369: 20-27
10. Carvalho FL, Simons BW, Eberhart CG, Berman DM: Notch signaling in prostate cancer: A moving target. *Prostate*, 2014; 74: 933-45
11. Yao WJ, Wang YL, Lu JG et al: MicroRNA-506 inhibits esophageal cancer cell proliferation via targeting CREB1. *Int J Clin Exp Pathol*, 2015; 8: 10868-74
12. Torre LA, Siegel RL, Jemal A: Lung cancer statistics. *Adv Exp Med Biol*, 2016; 893: 1-19
13. Han B, Park D, Li R et al: Small-molecule Bcl2 BH4 antagonist for lung cancer therapy. *Cancer Cell*, 2015; 27: 852-63
14. Fumarola C, Bonelli MA, Petronini PG, Alfieri RR: Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. *Biochem Pharmacol*, 2014; 90: 197-207
15. Dang TP: Notch, apoptosis and cancer. *Adv Exp Med Biol*, 2012; 727: 199-209
16. Alketbi A, Attoub S: Notch signaling in cancer: Rationale and strategies for targeting. *Current Cancer Drug Targets*, 2015; 15: 364-74
17. Radha G, Raghavan SC: BCL2: A promising cancer therapeutic target. *Biochim Biophys Acta*, 2017; 1868: 309-14
18. Polivka J Jr, Janku F: Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacol Ther*, 2014; 142: 164-75
19. Wang S, Lei Y, Cai Z et al: Girdin regulates the proliferation and apoptosis of pancreatic cancer cells via the PI3K/Akt signalling pathway. *Oncol Rep*, 2018; 40: 599-608
20. Wang LH, Yang JY, Yang SN et al: Suppression of NF-kappaB signaling and P-glycoprotein function by gambogic acid synergistically potentiates adriamycin -induced apoptosis in lung cancer. *Curr Cancer Drug Targets*, 2014; 14: 91-103
21. Qi Q, Lu N, Wang XT et al: Anti-invasive effect of gambogic acid in MDA-MB-231 human breast carcinoma cells. *Biochem Cell Biol*, 2008; 86: 386-95
22. Wang LH, Li Y, Yang SN et al: Gambogic acid synergistically potentiates cisplatin-induced apoptosis in non-small-cell lung cancer through suppressing NF-kB and MAPK/HO-1 signalling. *Br J Cancer*, 2014; 110: 341-52

Conclusions

We found that the Notch signaling pathway, specifically Notch/Bcl2 and Notch/PI3K signaling, was activated in human NSCLC cells. GA treatment inhibited the activation of Notch/Bcl2 and Notch/PI3K signaling, resulting in reduced expressions of Bcl2 and PI3K, which further induced apoptosis of NSCLC cells.