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Original Article

Genistein promotes apoptosis of lung cancer cells through the IMPDH2/AKT1 pathway

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Abstract: Objective: Lung cancer (LC) is a clinically challenging cancer. Genistein is a natural isoflavone product with anti-tumor effects. This study aims to investigate the effect of genistein on A549 cell apoptosis, to provide more experimental evidence for clinical treatment. Methods: Real-time quantitative polymerase chain reaction, western blotting, molecular docking, and target prediction methods were performed to detect the effect of genistein on LC cells. Cell viability of A549 treated by genistein was measured by a CCK-8 assay. The A549 cell apoptosis after genistein treatment was detected by flow cytometry. Results: Genistein promoted the apoptosis of LC cells in a time- and concentration-dependent manner. In addition, the low expression of inosine monophosphate dehydrogenase-2 (IMPDH2) inhibited the effect of genistein on LC cells. By predicting IMPDH2 LC-related apoptosis genes and finding the closely related gene protein kinase B (AKT1), it was found that the highly expressed AKT1 inhibited the effect of genistein on LC cell apoptosis and viability. Conclusion: Genistein may be a promising treatment for LC.

Keywords: Lung cancer, genistein, IMPDH2, AKT1 pathway, apoptosis

Introduction

Lung cancer (LC) has an increasing morbidity and mortality, especially in developing countries such as China [1, 2]. The GLOBOCAN 2008 cancer report shows 1.61 million new cases and 1.38 million deaths globally in 2008. The number of new cases ranks fourth (lower than breast, colorectal and cervical cancer), and mortality ranks second (after breast cancer) [3].

Genistein is an isoflavone in soybeans. Genistein plays a vital role in the prevention and treatment of common diseases by inhibiting inflammation, promoting apoptosis, regulating steroid hormone receptors and metabolic pathways in carcinogenesis, tumor spread, obesity, osteoporosis and metabolic syndrome [4]. Genistein is a key chemical component in the maintenance, prevention and treatment of diseases associated with metabolic syndrome and cancers [5]. The molecular mechanisms of genistein affecting carcinogenesis include its regulation of inflammation, cell proliferation and epigenetic changes. The cyclooxygenase-2 (COX-2) pathway is a potential target for genistein chemoprevention [6]. Genistein causes down-regulation of cyclinB1 (CCNB1), and it targets proteins involved in G2/M checkpoints (cell division cycle protein 2 (Cdc2) and cyclin-dependent kinase 1 (Cdk1)) in many cancers, such as breast cancer and prostate cancer [7, 8]. Genistein can regulate CCNB1, Cdc2, and other molecules related to cell cycle arrest and reduce the progression of tumor cells [9].

inosine monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme, and the enzymatic oxidation of nicotinamide adenine dinucleotide (NAD^+) is dependent on the oxidation of inosine monophosphate (IMP) to xanthine monophosphate (XMP), which is a key step in the biosynthesis of guanine nucleotides [10]. Human IMPDH consists of two different isomers, IMPDH1 and IMPDH2, with amino acid sequence similarity up to 84% [11]. IMPDH1 is commonly expressed in normal human white blood cells and lymphocytes, while IMPDH2 is up-regulated in tumor tissues and proliferating cells [12-14]. Moreover, the increase in the total IMPDH activity is mainly due to increased

IMPDH2 expression [15]. At present, the isomers of IMPDH, especially IMPDH2, are of great interest to oncologists in regulating cell proliferation and differentiation as well as affecting drug resistance [16-18].

The cell survival oncoprotein AKT, also named protein kinase B, is involved in pathophysiological processes that promote cell survival, proliferation, growth and migration [19]. AKT is often overactivated in human cancers. Oncogenic stimuli and growth factors can induce AKT kinase activity and promote anti-apoptotic signals in mammalian cells. Moreover, excessive activation of AKT is associated with poor prognosis and treatment resistance of human tumors [20, 21].

Effects of genistein in the progression of LC were explored in the study. Furthermore, our research explored whether genistein induces the apoptosis of LC cells via IMPDH2/AKT1 pathway. This study may provide a basis for the application of genistein in LC treatment in the future, and a new clue and theoretical basis for LC occurrence and prevention.

Materials and methods

Cell lines and cultures

Human lung adenocarcinoma epithelial cells (A549) were purchased from the American Type Culture Collection and cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific Company) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C. In the experiment, 0.05% trypsin-EDTA was used to harvest the cells under 80-90% confluence.

Cell counting kit (CCK-8)

A549 cells (1×10^4) were cultured in a 96-well plate at 37°C for 24, 48 and 72 h. 10 μl CCK-8 solution (Gibco; Thermo Fisher Scientific Company) was added and cultured for another 2 h. The absorbance was measured at 450 nm with a microplate automatic reader.

Flow cytometric analysis of apoptosis

A549 cells (1×10^4) were cultured for 72 h. Cells were collected and centrifuged at 12,000 × g for 5 min at room temperature, and resuspended in PBS after the supernatant was discarded. Cells were stained with Annexin-V-FITC and propidium iodide for 20 min at room temperature according to the instruction of AnnexinV-FITC cell apoptosis detection kit (Gibco; Thermo Fisher Scientific Company). Flow cytometry (LSRFortessa X-20; Becton, Dickinson and Company) was used to analyze the early and late apoptosis. The formula was the apoptosis rate (%) = the percentage of early apoptotic cells + the percentage of late apoptotic cells.

Reverse transcription quantitative polymerase chain reaction (qPCR)

Total RNA from cells was extracted with TRIzol reagents (Invitrogen; Thermo Fisher Science, Inc.). Total RNA was reverse transcribed into cDNA with SuperScript Reverse Transcriptase Kit (Thermo Fisher Science, Inc.) at 37°C for 15 min. Subsequently, two-step qPCR and CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Inc.) were performed using SYBR Green (Takara Bio, Inc.). The adopt thermal cycling conditions were as the following: 40 cycles, 95°C denaturation for 30 seconds, 60°C annealing for 30 seconds, 95°C extension for 10 seconds, and 65~95°C extension for 5 seconds. The primers were as follows: IMPDH2 forward, 5'-GTTTCTGGTATCCCAATC-3' and reverse, 5'-CGAGAGTCCAGCCTAT-3'; β-actin forward, 5'-TCATCATTGGCAATAG-3' and reverse, 5'-CACTGTGTTGGCGCATACAGGT-3'. β-actin was used as a normal control.

Predicting the target of genistein on LC cells

The common targets of LC and genistein were screened by the ctdbase.org database (http://ctdbase.org/). It was an innovative digital ecosystem that combines chemicals, genes, phenotypes, and diseases. The coexpedia coexpression database (https://www.coexpedia.org/) was used to analyze the interacting genes and select the genes that play an important regulatory role. The String online tool (https://string-db.org/) was used to predict the relationship between this important gene and NSCLC apoptosis-related genes, and to explore its main role in regulating genes. The metascape tool (https://metascape.org/gp/index.html) was used to perform Gene Ontology (GO) functional pathway enrichment analysis on the predicted common targets, and screen the targets involved in pathways in cancer and response to toxic substance.

Construction of molecular docking model

According to the chemical formula of genistein, we constructed its chemical structure model. The Swissmodel online tool (https://swissmodel.expasy.org/) and PyMOL online tool (https://pymol.org/2/) were used to compare the genes that played an important regulatory role in the interacting genes, and then the Autoduck tool (http://autodock.scripps.edu/) was used to calculate the genistein molecule and the important regulatory role of the interacting genes. The binding possibility of the protein molecule was the possible binding mode of the two.

Western blot and Co-IP

The same amount of protein was incubated with monoclonal antibody. The protein immune complex was precipitated by protein G-Sepharose, and then immunoblotting was performed with specific polyclonal antibody. In the presence of ExactaCruz reagent to reduce the IgG background, an IP-western analysis of gene-centromere correlation was performed using an anti-centromeric antibody against IP and a gene full-length polyclonal antibody for Western blotting. Also, for western blotting, the immunoprecipitation or cell lysate was dissolved in a 10% SDS-PAGE and then transferred to a nitrocellulose filter. The blot was incubated with the primary antibodies, and then incubated with a secondary antibody that matched the peroxidase-conjugated species. The bands were washed with TBST for 3 times × 10 min, and ECL chemiluminescence reagent was added. The spectral band intensity was analyzed automatically by Quantity One software.

Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis. Measured data were expressed as $\bar{x}\pm s$, and inter-group comparison was performed by t test. $P<0.05$ was considered significant.

Results

Genistein promotes LC cell apoptosis

The experiment used cisplatin as a control to explore how genistein affects LC cell apoptosis. **Figure 1A** shows that as the concentration of genistein increased, the viability of A549 cells decreased. Then, we found that compared to blank control, the rate of apoptosis increased with an increase of the concentration of genistein, which was similar to the result of cisplatin treatment (**Figure 1B**). Furthermore, expressions of cleaved-caspase-3 and cleaved-caspase-9 increased while caspase-3 and caspase-9 expressions decreased with increasing concentration (**Figure 1C**). This indicated that genistein could promote the apoptosis of LC cells.

The predicted common target of genistein and LC

A total of 255 results were obtained from the screening of common targets for LC and genistein on ctdbase.org (**Figure 2A**). **Figure 2B** displays the GO functional pathway analysis of the common 255 targets obtained on Metascape, and the targets involved in cancer and response to toxic substances pathways were screened. **Figure 2C** showed that IMPDH2 played an important regulatory role in gene interaction analysis by Coexpedia.

Molecular docking study of genistein and IMPDH2

The first constructed molecular model of genistein is shown in **Figure 3A**. Then, Swissmodel and PyMOL were used to construct the IMPDH2 molecular model (**Figure 3B**). Finally, Autoduck was used to calculate the possibility of stigmasterol molecules binding to RORC protein molecules. There were up to 10 possible combinations. **Figure 3C** exhibits the binding mode of some molecules (the light blue band represents protein molecules, and each color bar represents a binding mode of chemical molecules). The combination of genistein molecule and IMPDH2 did not promote the degradation of IMPDH2 ($P=0.5990$) (**Figure 3D**). IMPDH2 converted IMP ($P=0.0005$) into XMP ($P<0.0001$) during the guanosine monophosphate (GMP) synthesis process (**Figure 3E**). When the genistein drug molecule was combined, this process was accelerated, indicating that the combination with genistein promoted IMPDH2 activity.

Low expression of IMPDH2 inhibits the effect of genistein on LC cells

After IMPDH2 knockout, the viability was increased by comparison to that of the si-

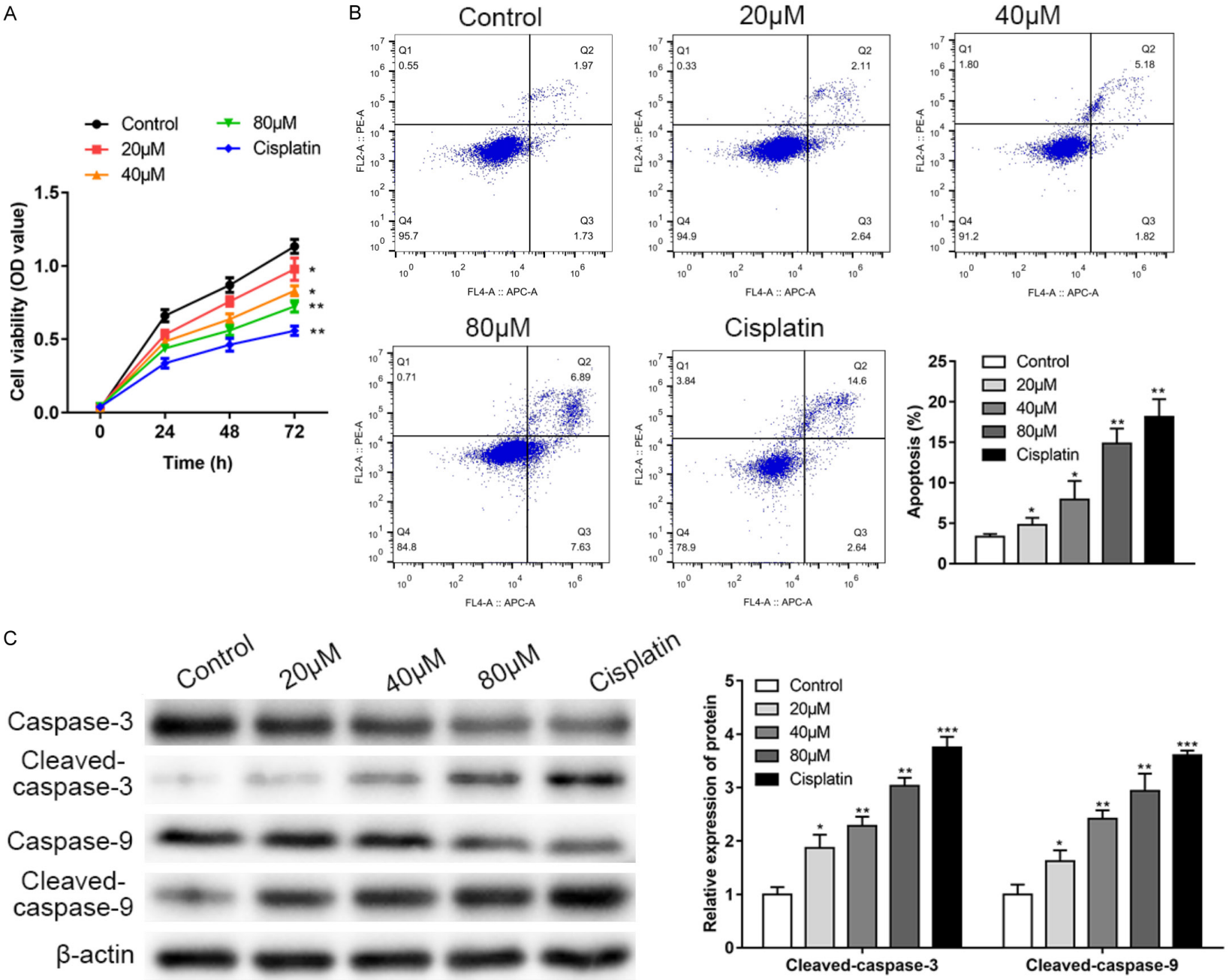
A
Control
20μM
40μM
80μM
Cisplatin
Cell viability (OD value)
Time (h)
B
Control
20μM
40μM
80μM
Cisplatin
FL2-A :: PE-A
FL4-A :: APC-A
Apoptosis (%)
C
Control
20μM
40μM
80μM
Cisplatin
Caspase-3
Cleaved-caspase-3
Caspase-9
Cleaved-caspase-9
β-actin
Relative expression of protein
Cleaved-caspase-3
Cleaved-caspase-9

Figure 1. Genistein promotes lung cancer cell apoptosis. A: CCK-8 was used to measure cell viability at different genistein concentrations; B: Flow cytometry was used to measure cell apoptosis at different genistein concentrations; C: Western blot was used to verify cell apoptosis-related proteins. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

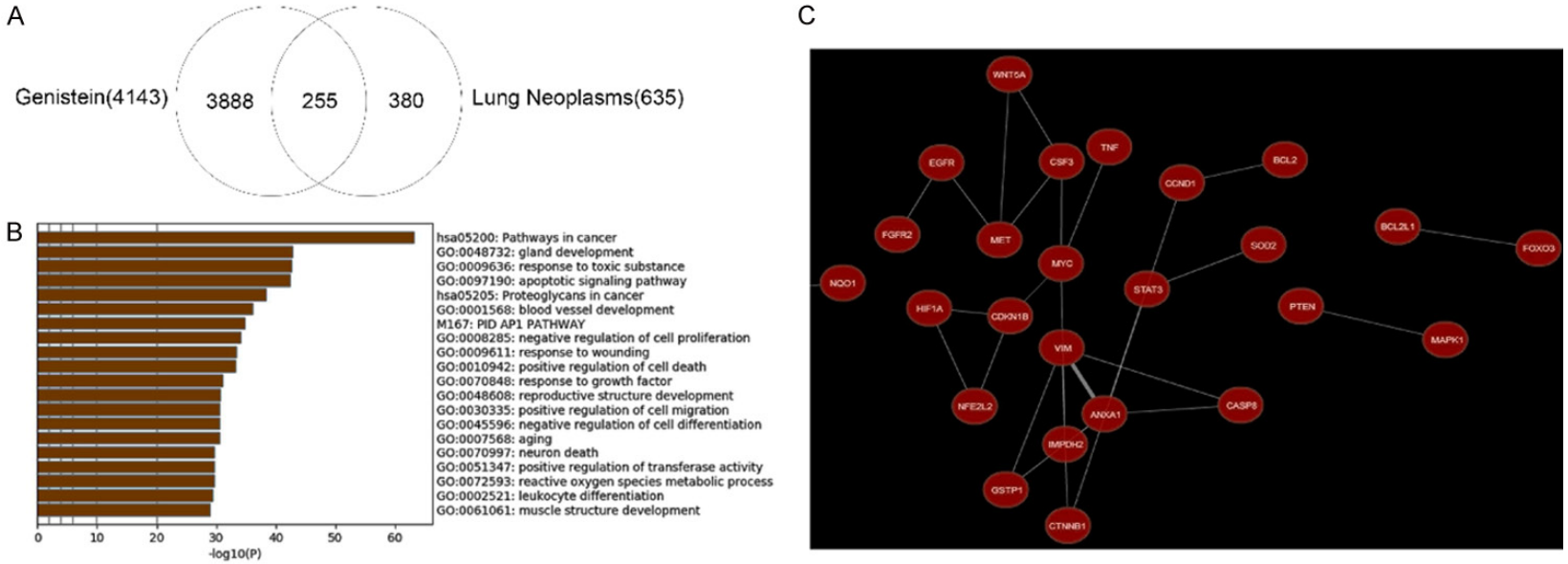

Figure 2. Predicting the common target of genistein and lung cancer. A: A total of 255 targets were screened on ctdbase.org; B: Gene Ontology functional pathway analysis was performed on the common 255 targets; C: The coexpedia analysis were carried out for gene interaction verification.

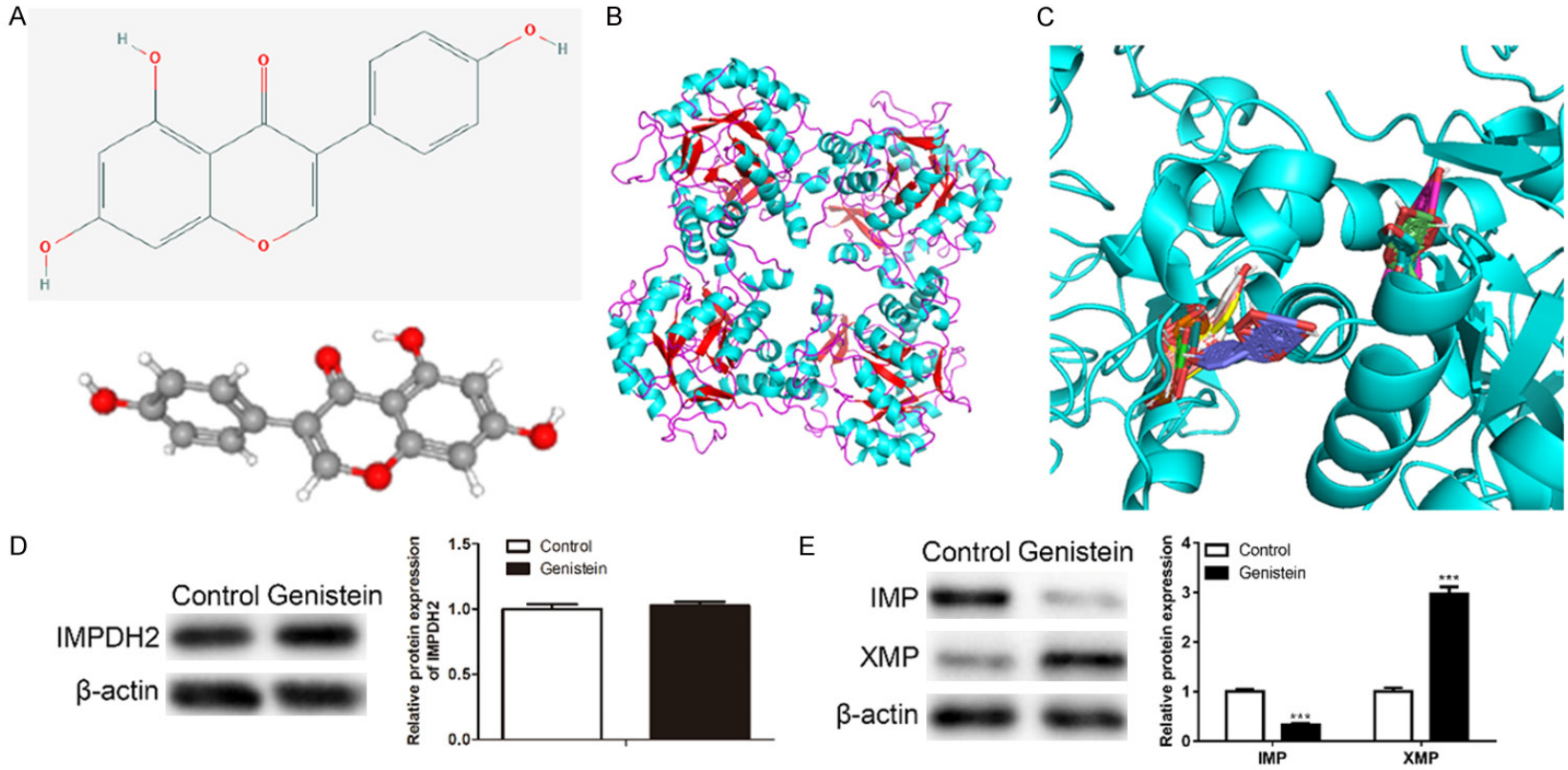

Figure 3. Molecular docking of genistein and IMPDH2. A: Construction of the chemical structure of genistein; B: Swissmodel and PyMOL constructed IMPDH2 molecular model; C: Autoduck calculated molecular binding; D: The change in IMPDH2 expression after genistein treatment (detected by western blot); E: Western blot was used to detect the effect of genistein on the process of converting IMP to XMP. ***P<0.001.

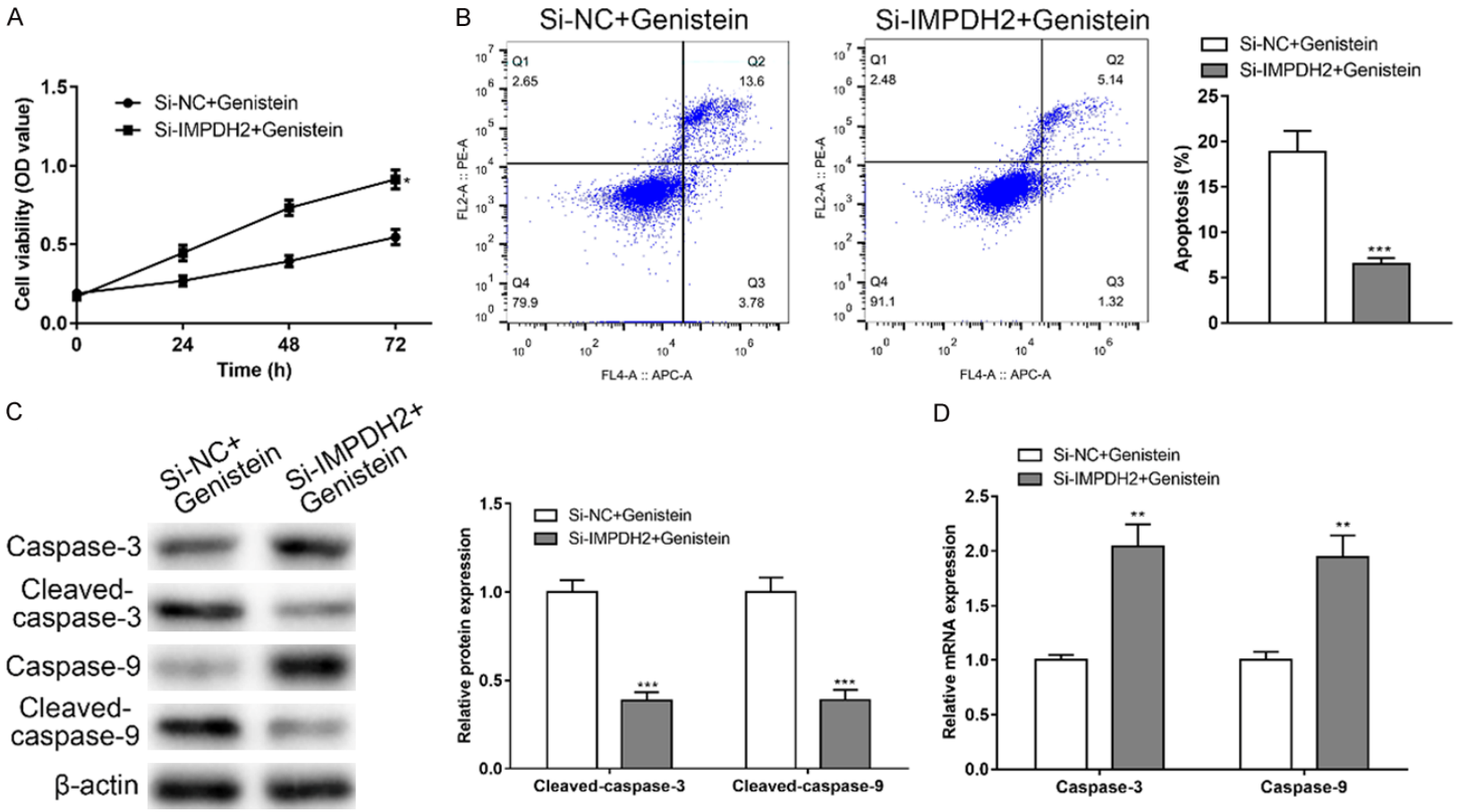

Figure 4. Low expression of IMPDH2 inhibits the effect of genistein on lung cancer cells. A: CCK-8 detected the effect of IMPDH2 inhibition on the viability of A549 cells treated with genistein; B: The effect of IMPDH2 inhibition on the apoptosis of A549 cells treated with genistein was detected by flow cytometry; C, D: Western blot and qPCR verified the effect of IMPDH2 inhibition on protein and mRNA expressions in A549 cells treated with genistein. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

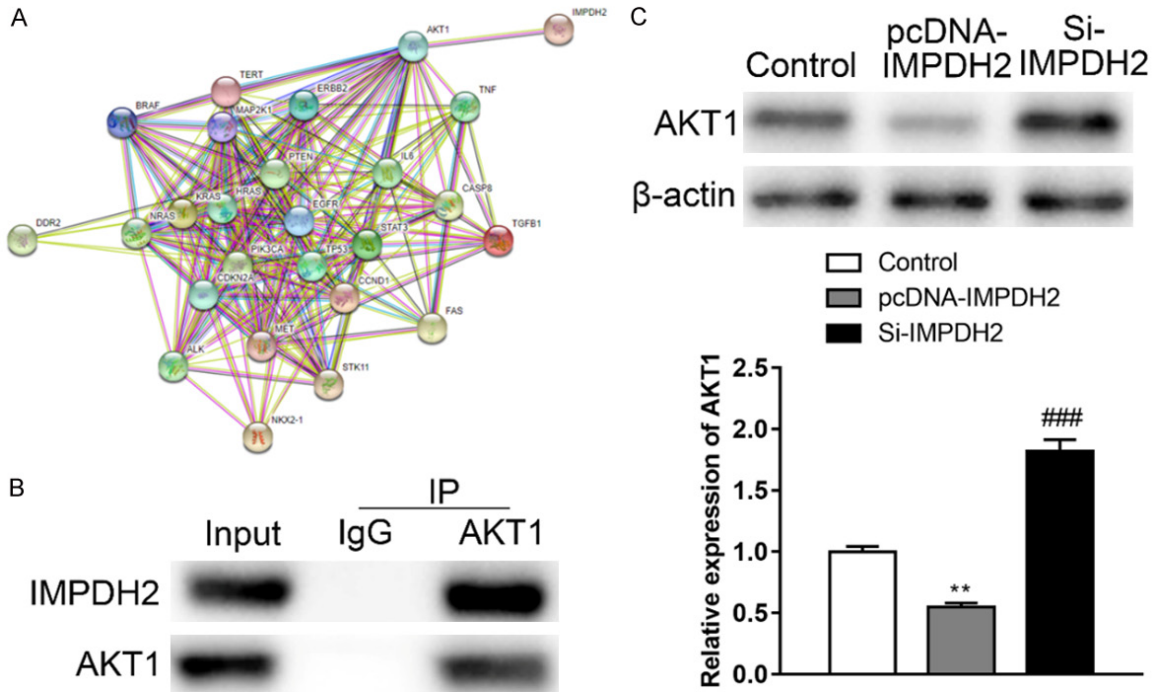

Figure 5. IMPDH2 regulates the apoptosis pathway gene AKT1. A: String predicted IMPDH2 and NSCLC apoptosis-related genes; B: Co-immunoprecipitation technology detected the relationship between IMPDH2 and AKT1; C: Western blot verified the effect of IMPDH2 on AKT1 expression. $^{**}P<0.01$, $^{###}P<0.001$.

NC+genistein group (P=0.0164) (**Figure 4A**), indicating that low expression of IMPDH2 could inhibit the effect of genistein on A549 cells. The flow cytometry results found that when IMPDH2 was knocked down, cell apoptosis was significantly reduced compared to that of the si-NC+genistein group (P=0.0009) (**Figure 4B**). When IMPDH2 was expressed at a low level, cleaved-caspase-3 and cleaved-caspase-9 expressions were significantly reduced (P< 0.0001; P<0.0001), and caspase-3 (P= 0.0012) and caspase-9 expressions were significantly increased (P=0.0017) (**Figure 4C**, **4D**). Therefore, effects of genistein on A549 cell viability and apoptosis were significantly inhibited when IMPDH2 was lowly expressed.

Prediction and verification of IMPDH2 regulating apoptosis pathway gene AKT1

Using String to predict the relationship between IMPDH2 and NSCLC apoptosis-related genes, it was found that it mainly acted on AKT1 (**Figure 5A**). IMPDH2 could bind to AKT1 by immunoprecipitation technology (**Figure 5B**), and western blot further found that the protein molecule binding of IMPDH2 and AKT1 might promote the degradation of AKT1 (**Figure 5C**).

Overexpression of AKT1 inhibits the effect of genistein on LC cells

Overexpression of AKT1 could reverse the effect of genistein on A549 cell viability (P= 0.0205) and apoptosis (P=0.0007) (**Figure 6A**, **6B**). Moreover, western blot and qPCR verified that when AKT1 was overexpressed, cleaved-caspase-3 and cleaved-caspase-9 expressions were significantly reduced (P<0.0001), and caspase-3 and caspase-9 expressions were significantly increased (P=0.0122; P=0.0024) (**Figure 6C**, **6D**). Therefore, when AKT1 was overexpressed, the effect of genistein on A549 cells was significantly inhibited.

Discussion

The effect of genistein on A549 cells was investigated. Results suggested genistein promoted lung cancer (LC) cell apoptosis through the IMPDH2/AKT1 pathway. Therefore, this study suggested that genistein could be used as a treatment for LC.

Genistein has the effect of preventing cancer. Bao et al. proved that FOXM1 overexpression reversed the inhibitory effect of genistein on

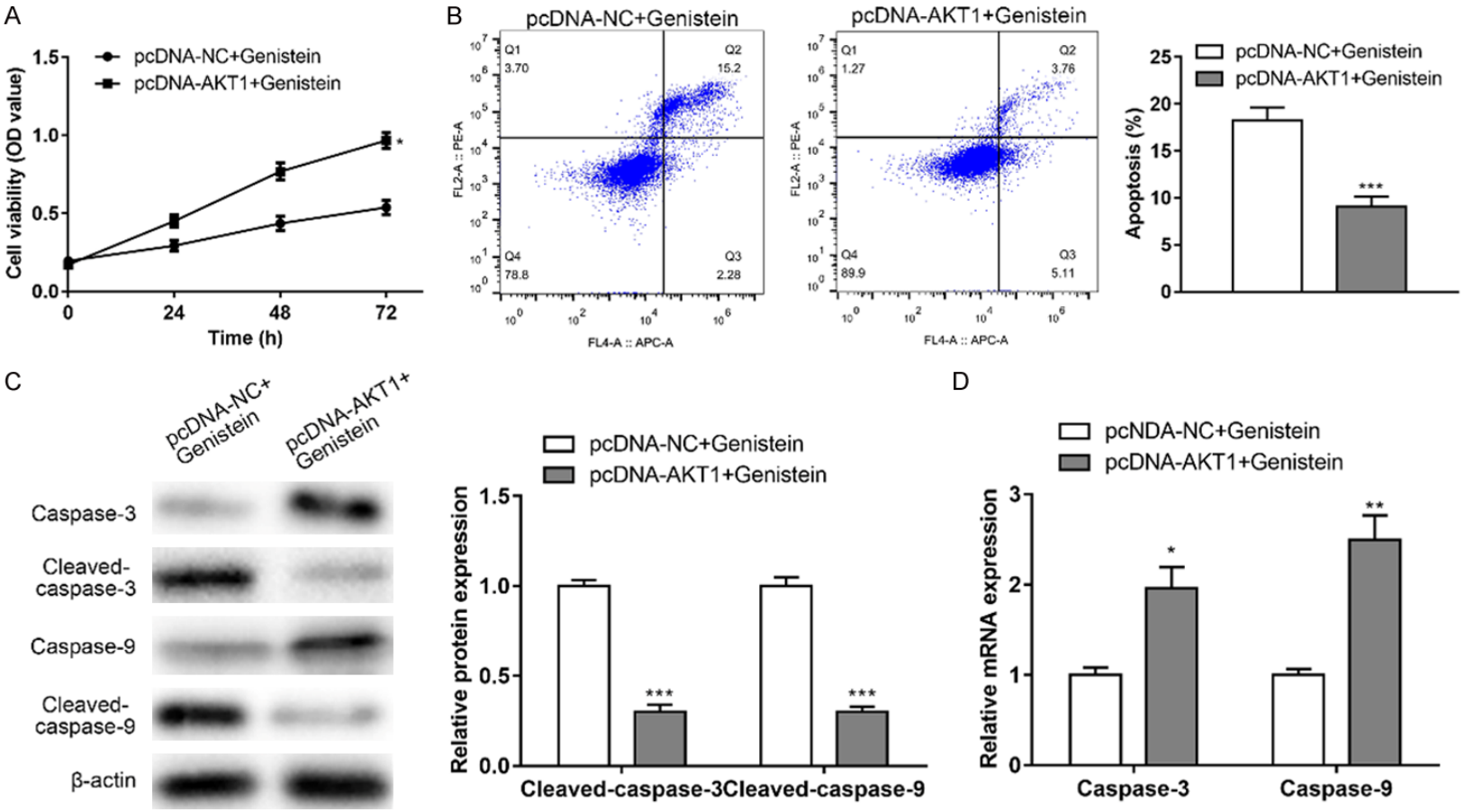

Figure 6. Overexpression of AKT1 inhibits the effect of genistein on lung cancer cells. A: CCK-8 detected the effect of AKT1 overexpression on the viability of A549 cells treated with genistein; B: The effect of AKT1 overexpression on the apoptosis of A549 cells treated with genistein was detected by flow cytometry; C, D: Western blot and qPCR verified the effect of AKT1 overexpression on protein and mRNA expressions in A549 cells treated with genistein. $^{**}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

CSC characteristics [22]. Huang et al. reported that the chemotherapeutic sensitization caused by genistein in gastric cancer cells was related to the inhibition of ERK1/2 activity [23]. Genistein could further inhibit the formation of stem cell-like cells in MCF-7 cells by down-regulating the Hedgehog-Gli1 pathway [24]. In addition, it was shown that genistein promoted colorectal cancer cells apoptosis by inhibiting the NF-κB pathway [25]. This suggested that the anti-cancer effect of genistein was related to multiple signaling pathways. Our results also found genistein induced apoptosis and reduced viability of LC cells.

IMPDH2 is considered an important target for tumor therapy. Many studies have shown that IMPDH2 is involved in cell proliferation and tumorigenesis [26-28]. Since 1975, IMPDH has been implicated in cell proliferation and malignancy [29]. Increasing evidence has indicated that IMPDH2 associates with different types of malignant tumors [11, 18, 30, 31]. In addition, IMPDH2 may serve as a prognostic biomarker for patients [11]. IMPDH2 is also an effective immunosuppressant target, and IMPDH inhibitors have been researched and developed for tumor suppression [32-34]. Here, we found that IMPDH2 was up-regulated in A549 cells, which is consistent with a previous study [35]. When IMPDH2 was lowly expressed, the inhibition of genistein on LC cells viability was reversed. Therefore, the interaction of genistein and IMPDH2 led to cell apoptosis and decreased the viability of LC cells.

The PI3K/AKT/mTOR pathway regulates cell growth, adhesion, migration, survival and other cellular events [36-39]. Activation of AKT signaling may promote cell proliferation and tumor progression by regulating its downstream cell cycle factors [40]. This study showed that AKT1 overexpression inhibited the effect of genistein on LC cells, indicating opposite effects. Our findings revealed that IMPDH2 was overexpressed, which limited the Akt signaling pathway and induced tumor cell apoptosis. In short, the anti-cancer effect of genistein was realized by the regulation of IMPDH2 and AKT1 pathways. This showed that genistein might be an auxiliary drug for LC treatment. However, the role of genistein in preclinical animal models of LC remains to be studied.

Disclosure of conflict of interest

None.

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