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ORIGINAL RESEARCH

Xingxiao Pill Suppressed the Progression of Non-Small Cell Lung Cancer by Targeting SREBP1/FASN–Induced Fatty Acid Biosynthesis via PI3K/AKT/mTOR Signaling Pathway

Xiangnan Zhou[1,2,*], Xiuhua Hu[2,3,*], Zhiying Zhang[2,*], Shicheng Lin[2], Ximing Lin[2], Tian Zhou[2], Yanping Bai[1], Kaiwen Hu[2]

[1]Department of Dermatology, China-Japan Friendship Hospital, National Center for Integrated Traditional Chinese and Western Medicine, Beijing, 100029, People's Republic of China; [2]Department of Oncology, East Hospital, Beijing University of Chinese Medicine, Beijing, 100078, People's Republic of China; [3]School of Life Sciences, Beijing University of Chinese Medicine, Beijing, 102488, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yanping Bai, China-Japan Friendship Hospital, National Center for Integrated Traditional Chinese and Western Medicine, Beijing, 100029, People's Republic of China, Email yanpbCJFH@163.com; Kaiwen Hu East Hospital, Beijing University of Chinese Medicine, Beijing, 100078, People's Republic of China, Email kwhbucm@163.com

Introduction: Xingxiao Pill (XXP), a typical traditional Chinese medicine (TCM) prescription drug used to treat NSCLC in clinic. However, the mechanism underlying its regulatory effects remains unclear. This study aimed to evaluate the potential efficacy of XXP in treating NSCLC and to investigate how XXP regulates fatty acid biosynthesis in NSCLC.

Methods: A lung carcinoma mouse model was created by transplanting Lewis lung carcinoma (LLC) cells into male C57BL/6 mice. Lung cancer cell models using LLC and A549 cells were also constructed. XXP's therapeutic efficacy on NSCLC was assessed via oral gavage. Bioinformatics analysis and transcriptome sequencing identified XXP's potential targets and mechanisms. These findings were verified by in vitro cell assays, Western blotting, immunofluorescence staining, and Oil Red O staining.

Results: XXP inhibited lung tumor growth, suppressed cell proliferation and impeded cell migration. Additionally, it influenced the processes of apoptosis and cell cycle in both A549 and LLC cells. Bioinformatics analysis suggested that regulation of fatty acid biosynthesis and phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway were crucial mechanisms underlying the antitumor effects of XXP in lung cancer. XXP reduced the levels of the fatty acid biosynthesis products, such as total cholesterol (TC), triglycerides (TG), lipids, and free fatty acids in A549 cells, and downregulated the expression of sterol regulatory element binding protein 1 (SREBP1) and fatty acid synthase (FASN). Furthermore, XXP decreased the expression level of PI3K, AKT, mTOR, phospho–PI3K, and phospho–AKT.

Discussion: XXP exerts its inhibitory effect on lung cancer tumor growth by controlling the biosynthesis of fatty acids and the PI3K/AKT/mTOR signaling pathway. The research suggests that targeting this metabolic pathway could be a viable strategy for cancer therapy and emphasizes the value of TCM in providing a rich source of innovative pharmaceuticals for cancer treatment.

Keywords: Xingxiao Pill, PI3K/AKT/mTOR pathway, fatty acid biosynthesis, lung cancer, SREBP1, FASN

Introduction

Globally, lung cancer tops the list for both incidence and fatality, recording 2.2 million new diagnoses and 1.8 million deaths in 2020.[1] In China, the latest figures from the National Cancer Center for 2022 reveal that lung cancer continues to be the foremost malignancy in terms of both occurrence and mortality. It is predominantly divided into two types: small cell lung cancer and non-small cell lung cancer (NSCLC), with the latter being significantly more common, representing 85% of lung cancer diagnoses.[2] Even with progress in treatments including surgery, chemotherapy, and immunotherapy, the prognosis for lung cancer, particularly NSCLC, is still generally poor due to factors such as late-stage diagnosis, metastasis, and the development of resistance to therapies.[3]

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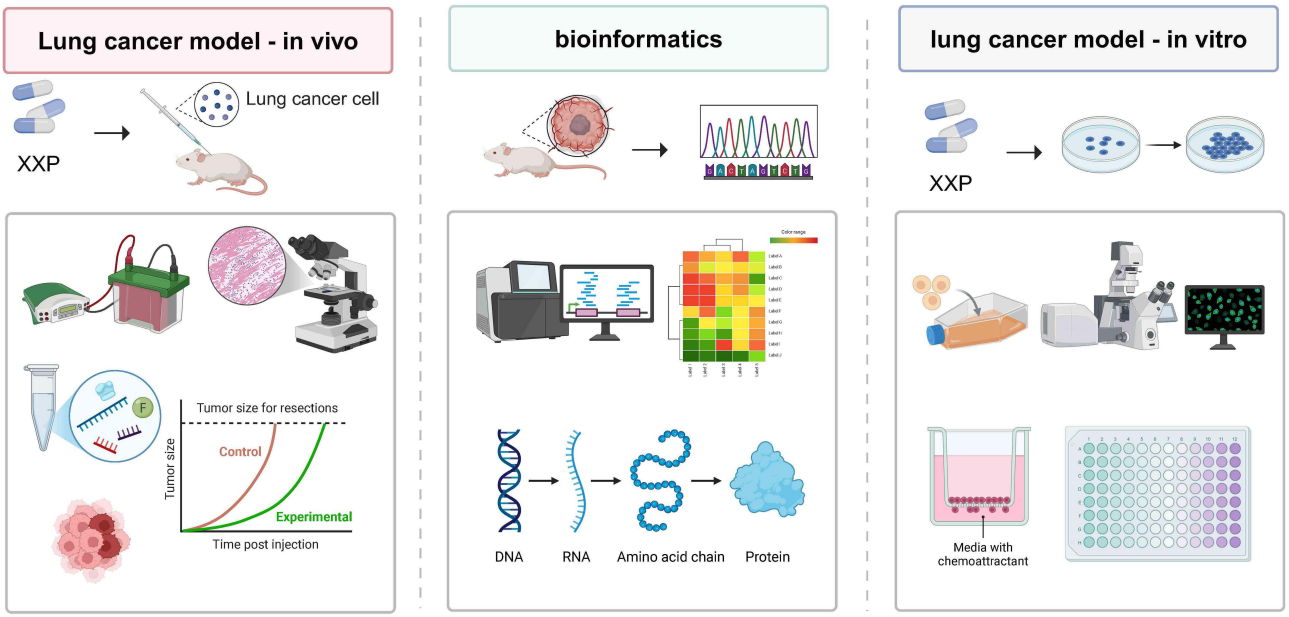
Graphical Abstract



TCM has been demonstrated the ability to both amplify the efficacy of current treatments and concurrently elevate the life quality for those afflicted with lung cancer.[4,5] Xingxiao Pill (XXP), a classic TCM prescription drug, is composed of *Realgar* (As_2S_2 or As_4S_4),[6] *Moschus*,[7] *Boswellia sacra* Flück., and *Commiphora myrrha* (T.Nees) Engl., and is known to improve clinical discomfort, regulate metabolism, and ensure the living standards of individuals affected lung cancer.[8] Moreover, experimental studies have reported XXP's potential in treating lung cancer by antitumor immunity and inhibiting tumor angiogenesis.[9,10] Moreover, it has been shown to have therapeutic effects on nodular goiter by modulating cell apoptosis and proliferation.[11]

Recently, the metabolism of fatty acids in cancer cells has become a focal point of interest, which serves as a fundamental resource for the swift multiplication of cancer cells and also furnishes the ample energy necessary to sustain their aggressive growth.[12] Abnormal fatty acid metabolism in lung tissue is closely related to NSCLC occurrence and pathogenesis. Crucial enzymes and regulatory factors within fatty acid metabolism are instrumental in driving cancer cell growth, viability, and the spread of the disease.[13]

TCM holds promise as a preventive strategy against cancer and is capable of modulating fatty acid metabolism in cancer cells. Consequently, the objective of this research was to explore the mechanisms by which XXP influences fatty acid metabolism in NSCLC through both in vivo and in vitro analyses.

Materials and Methods

Drugs

XXP (SFDA [Saudi Food and Drug Authority] approval number Z11020073, lot number 18041283) was obtained from Beijing TRT Group (Beijing Tong Ren Tang Group, Beijing, China). XXP is composed of *Realgar* (As_2S_2 or As_4S_4),[6] *Moschus*,[7] *Boswellia sacra* Flück., and *Commiphora myrrha* (T.Nees) Engl.

The XXP dosage was determined to be 6 g/d in accordance with the somatotype coefficient for adult humans and mice was 12.33,[14] with an assumed average adult body weight of 60 kg. Mice of lung carcinoma were divided into three dosage groups: middle dose group (M–XXP; 1.233 g/[kg·d]), low dose group (L–XXP; 0.617 g/[kg·d]), and high dose group (H–XXP; 2.466 g/[kg·d]) described in detail below. For the in vitro assay, 5 g XXP was immersed in 50 mL dulbecco's modified eagle medium for a period of 24 h (crude concentration 100 mg/mL), followed by ultrasonication for two hours. After a 48-hours soak, the supernatant was collected and filtered through a 0.22 μm membrane.[15]

Characterization of the Components in XXP by UPLC-Q-Orbitrap HRMS

1.0 g of XXP sample and 40 mL of 80% methanol were ultrasonicated for 30 min in a 50 mL tube, then centrifuged at 4°C, 12,000 rpm for 10 min. 100 μL of supernatant was used for detection. Separation was conducted on a Vanquish Flex UHPLC system with an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 μm). The mobile phase was water (0.1% formic acid, A) and acetonitrile (B) at 0.3 mL/min, with a column temperature of 40°C. Mass spectrometry data were collected using a Q Exactive mass spectrometer with a HESI-II probe. Settings included ion source voltages of 3.7 kV (positive) and 3.5 kV (negative), a heated capillary temperature of 320°C, sheath gas pressure of 30 psi, auxiliary gas pressure of 10 psi, and a desolvation temperature of 300°C. All gases were nitrogen, with a collision gas pressure of 1.5 mTorr. Data were acquired in "full scan/dd-MS²" mode.

Generation of a Mouse Model of Lung Carcinoma

The LLC cell line (batch number KG070) was procured from Jiangsu KeyGEN BioTECH Co., Ltd., a company based in Nanjing, China. Male C57BL/6 mice, aged 4–6 weeks, weighing 18–22 g, were sourced from SPF (Beijing) Biotechnology Co Ltd (Beijing, China). LLC cells were counted and adjusted into a single cell suspension at a concentration of 5×10^6 cells/mL. C57BL/6J mice were subcutaneously injected with 0.2 mL of the cell suspension under the armpit of their right forelimb to establish a Lewis lung cancer-bearing model.[16] They were categorized into five groups: the model control (MC), L–XXP, M–XXP, H–XXP, alongside a positive control group treated with cisplatin (Qilu Pharmacy Co., Ltd., approval number H37021358). All procedures involving animals were conducted following the ethical guidelines of the Welfare and Ethics for Laboratory Animals, as stipulated by the Institutional Animal Care and Use Committee of SPF Biotechnology Co., Ltd., with the document number AW2020111001. Once the lung carcinoma mouse model was established, the lung tumor tissues were excised and then processed for hematoxylin and eosin (H&E) staining.

Bioinformatics Analysis

The pharmacological targets and constituent profiles of XXP were identified utilizing various TCM databases and analytical platforms, including the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP), bioinformatics analysis tool for molecular mechanism of traditional Chinese medicine (BATMAN-TCM), encyclopedia of traditional Chinese medicine (ETCM), and high-throughput experiment and reference-guided database of TCM (HERB). The GEO2R tool was employed to screen lung cancer–associated differentially expressed genes (DEGs) in the GSE116959 data within Gene Expression Omnibus. The ggplot2 package (The Comprehensive R Archive Network), along with Venn Diagram with R (RStudio), and Cytoscape, were utilized to acquire intersection targets. Subsequently, the protein-protein interaction (PPI) network and network analysis was arranged using CytoNCA, which facilitated the creation of a hub network from the intersecting targets and the selection of key targets. Additionally, the clusterProfiler package facilitated gene ontology (GO) functional annotation coupled with enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Transcriptome Sequencing

Tumor tissues were harvested from the MC and H–XXP group (n = 3 each) in the lung cancer xenograft mice, and they were subjected to transcriptome sequencing via RNA-Seq at BGI Genomics.

Cell Culture and Proliferation, Cell Cycle and, Apoptosis Assays

The A549 cell line, obtained from the National Infrastructure of Cell Line Resource Sharing (NICR, Beijing, China). LLC and A549 cells were cultivated in dulbecco's modified eagle medium, augmented with 10% fetal bovine serum (FBS), under conditions at 37°C. Cell proliferation was evaluated with CCK-8 (C0038. Beyotime). The PE Annexin V Apoptosis Detection kit (KGA1012, KeyGEN BioTECH, Jiangsu, China) and the Cell Cycle Detection Kit (KGA512, KeyGEN BioTECH) were utilized for analyzing apoptosis and cell cycle progression, respectively, employing flow cytometry on a CytoFLEX LX system.

Transwell Assay

The transwell method assessed cell migration, where cells were seeded in serum–free medium in the upper compartment, facing a lower chamber filled with medium supplemented with 10% fetal bovine serum. After 24 hours, the cells were treated with 4% paraformaldehyde for fixation, followed by staining with crystal violet, and then documented through photography.

Oil Red O Staining

Lipid droplets in the cells were identified through Oil Red O staining, following the protocol of an Oil Red O staining kit provided by Solarbio Life Sciences, Beijing, China. The procedure involved fixing the cells with the kit's fixative, applying the Oil Red O stain, and subsequently counterstaining the nuclei with Mayer's hematoxylin solution.

TC, TG, and Free Fatty Acid Content Analysis

The levels of TC, TG, and free fatty acid in A549 cells were measured using specific assay kits: the triglyceride and cholesterol determination kits (catalog numbers A110-1-1 and A111-1-1, respectively, from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the free fatty acid assay kit (BC0595, Solarbio Life Sciences).

Western Blotting

Proteins were extracted with a protein extraction kit (R0050, Solarbio, China). The processes of protein electrophoresis, membrane transfer, and blotting were conducted in line with established procedures.[17] During the analysis, we deployed a suite of antibodies, encompassing anti–AKT (10176-2-AP), anti–phospho–AKT (66444-1-Ig), anti–PI3K (60225-1-Ig), anti–mTOR (28273-1-AP), anti–FASN (10624-2-AP), and anti–SREBP1 (66,875-1-Ig), which were obtained from Proteintech located in Wuhan, China. Additionally, antibodies like anti–phospho–PI3K (17366) and anti–phospho–mTOR (5536) were procured from Cell Signaling Technology in Massachusetts, United States. Protein bands were visualized with a system for fluorescence molecular imaging (170–8280, Bio-Rad). With GAPDH serving as a reference control, the relative expression levels of proteins were determined by comparing the intensities of the bands for the proteins of interest to those of the reference.

Immunofluorescence Staining

Cell samples were collected and permeabilized, followed by blocking with normal goat serum (SL038, Solarbio). They were then stained with primary antibodies and fluorescent dye–conjugated secondary antibodies. Subsequently, the cell nuclei were labeled with DAPI. Fluorescent cellular images were captured utilizing a confocal microscope (FluoView FV1000, Olympus).

Statistical Analysis

Initially, all samples underwent the D'Agostino–Pearson test for normality. Gaussian-distributed data were analyzed using one-way ANOVA complemented by Bonferroni's post-tests for multiple comparisons. In cases of non-Gaussian distributions, the Kruskal–Wallis test was the method of choice for assessing differences across groups. Statistical evaluations were conducted with GraphPad Prism (9.5.0, United States).

Results

XXP Suppressed the Progression of NSCLC within the Animal Models

Figure 1A depicted the detailed experimental protocol and procedural steps of the in vivo experiments. We initially developed a NSCLC mouse model to assess the impact of XXP on tumor growth inhibition. Observations indicated that the weight of the NSCLC xenograft mouse models increased progressively with the administration of XXP (Figure 1B). Despite a consistent increase in tumor volume across all groups, the H–XXP group and positive control group exhibited reduced tumor sizes as compared to the MC group on day 12 and day 15 (Figure 1C). On day 15, the tumor growth rate was markedly reduced in both the H–XXP group ($P < 0.05$) and positive control group ($P < 0.001$), as contrasted with the MC group (Figure 1D). The tumor volume/body weight ratio was then served as an indicator to express the rate of tumor

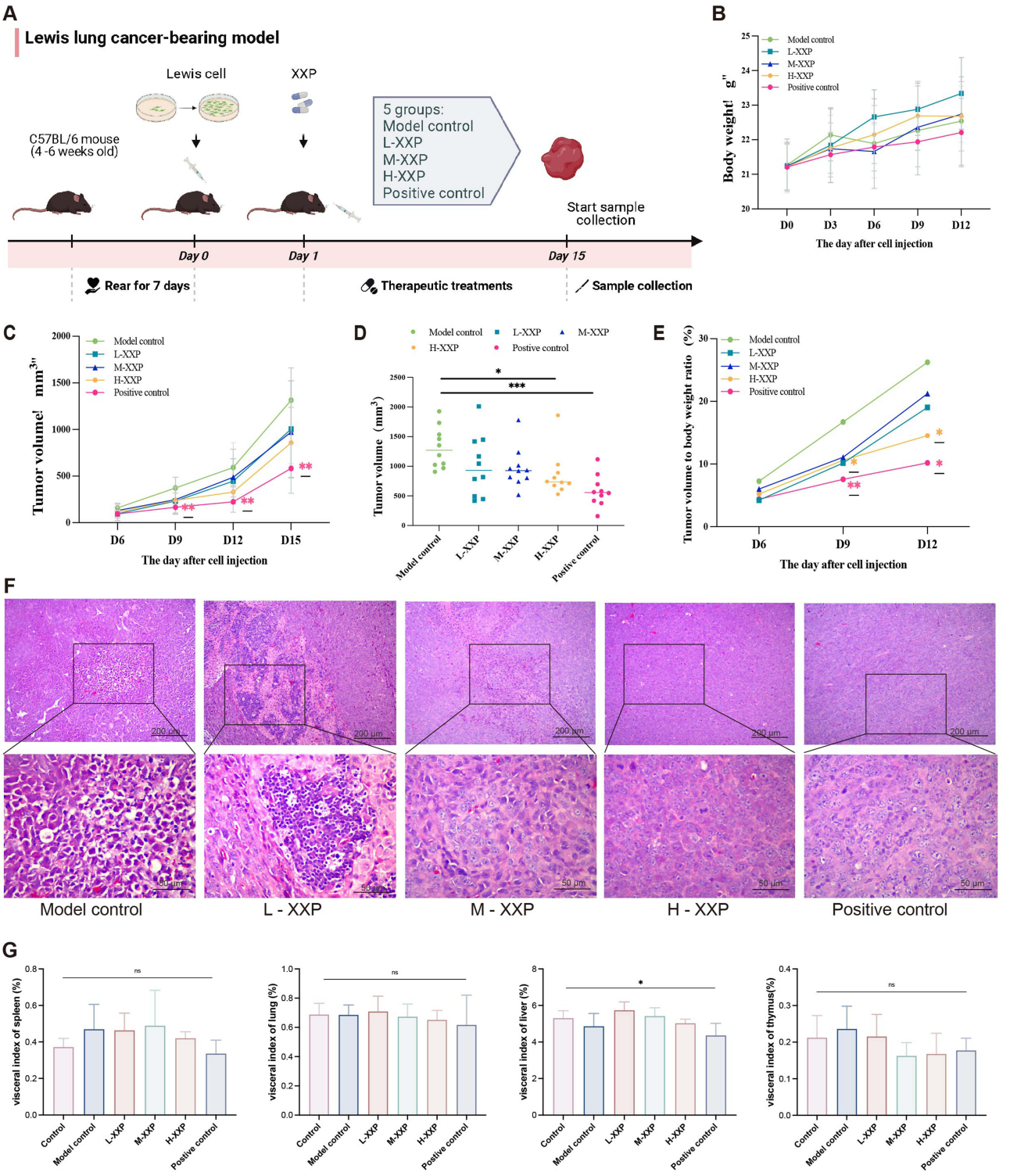


Figure 1 Effect of XXP treatment on NSCLC xenograft mouse models. (**A**) In vivo experimental procedure; (**B**) the body weight of mice from cell injection to the 12th day; (**C**) growth of the tumor volume in mice with time; (**D**) tumor volume of mice on the 15th day; (**E**) the tumor volume/ body weight ratio of mice on the 6th, 9th, and 12th days; (**F**) results of H&E staining of tumor tissues. (**G**) The percentage of visceral coefficient of mouse in each group. n = 10. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus the model control group.

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progression,[18] a reduction of which was noted in both the H–XXP and the positive control group on day 12, as contrasted with the MC group (Figure 1E). Additionally, H&E staining revealed that XXP–treated mice had smaller nuclei and a reduced nucleo–cytoplasmic ratio compared to the MC group. This was accompanied by a vacuolated appearance of tumor cells and a decrease in lymphocytic infiltration (Figure 1F). At the experimental endpoint, no significant differences were observed in the spleen index, lung index and thymus index across the different groups of mice. As for liver index, the organ index of the positive control group decreased compared with the control group (Figure 1G). Furthermore, our previous clinical trials had demonstrated that XXP is well-tolerated and exhibits good safety in the treatment of non-small cell lung cancer.[8] These findings provided evidence supporting the safety profile of XXP in the treatment of lung cancer. Collectively, these findings indicated the potential of XXP to exert antitumor activity in NSCLC xenograft mouse models.

XXP Restrained NSCLC Cell Proliferation, Induced Cell Apoptosis, Regulated Cell Cycle, and Impeded Cell Migration

The widespread consensus is that the progression of cancer is significantly propelled by processes including tumor growth, cell migration, invasion, and proliferation.[19] Regulating these factors within lung cancer cells and in murine models is crucial for the development of effective NSCLC treatments. We assessed the impact of XXP on the human lung cancer cell lines, A549 and LLC, through in vitro experiments, the experimental procedure is shown in Figure 2A. XXP reduced cell viability of A549 cells (Figure 2B) and LLC cells (Figure 2C) in a concentration-dependent fashion. The IC_{50} value of XXP intervened by A549 and LLC was 0.6527 mg/mL and 0.4650 mg/mL.

After treatment with XXP, the cell cycle of A549 cells (Figure 2D and E) and LLC cells (Figure 2F and G) was altered, with a decrease in the G1 phase and an increase in the S and G2 phases. This indicated that XXP affected the normal progression of the cell cycle in both cell lines, causing more cells to be arrested in the G2 phase. XXP appeared to have the ability to regulate the transition from the G1 to the G2 phase, thereby influencing cell proliferation. Interestingly, at the 25% inhibitory concentration (IC_{25}) and the 50% inhibitory concentration (IC_{50}), XXP could significantly ($P < 0.01$) induce apoptosis in A549 cells (Figure 2H and I). In contrast, although no significant apoptotic effect was observed in LLC cells (Figure 2J and K), we still observed a trend toward apoptosis in LLC cells after treatment with XXP. This suggested that XXP had a potential influence on inducing apoptosis in LLC cells, even if the effect did not reach statistical significance. Furthermore, XXP markedly inhibited the migratory capacity of both A549 (Figure 2L and M) and LLC cells (Figure 2N and O). These findings suggested that XXP possessed the ability to suppress the proliferation and migration of NSCLC cells in vitro, aligning with its observed antitumor activity in vivo studies.

Prediction of the Chemical Composition of XXP and Its Potential Underlying Mechanisms in Lung Cancer Treatment

Our earlier research employed UPLC-Q-Orbitrap HRMS to determine the chemical constituents of XXP (Figure S1A and S1B, Table S1). We found that the main components of XXP are Myrrhone, Poricoic acid B, 11-Keto-beta-boswellic acid. Myrrhone, a sesquiterpene compound extracted from Commiphora myrrha, exhibits a range of biological activities, including the ability to inhibit the proliferation of hepatocellular carcinoma cells,[20] skin cancer cells,[21] and renal cancer cells.[22] Poricoic acid B is a naturally occurring triterpenoid compound, known for its anticancer,[23] metabolism regulation[24] and anti-inflammatory[25] activities. 11-Keto-beta-Boswellic acid is a pentacyclic triterpenoid acid known for its anti-inflammatory,[26] anticancer,[27] and antioxidant properties. Studies have shown that it can induce apoptosis and inhibit the proliferation of prostate cancer cells.[28] These compounds are essential and associated with the antitumor effects of XXP. Moreover, we identified 66 active components of XXP through the network pharmacological analysis (Table S2), including androst-4-ene-3,17-dione, 3α-hydroxy-5α-androstan-17-one, β-boswellic acid, and α-boswellic acid. 3α-hydroxy-5α-androstan-17-one aids in distinguishing early-stage from advanced clear cell renal cell carcinoma.[29] Specifically, α- and β-boswellic acid demonstrate exceptional anti-inflammatory, anti-cancer, and antimicrobial properties,[30] thereby reinforcing the evidence for the anti-cancer potential of XXP.

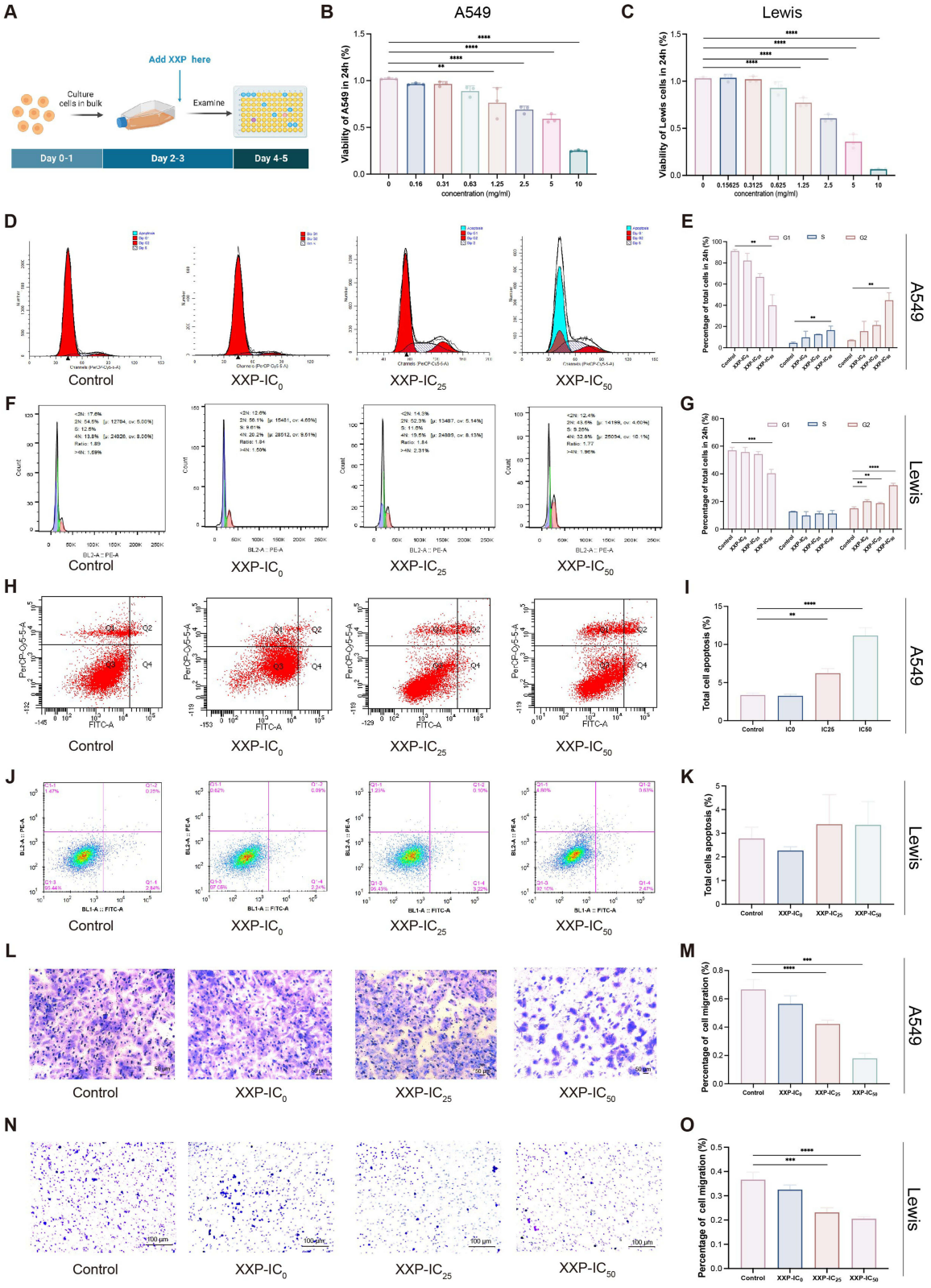

Figure 2 Results of the cell proliferation, cell cycle and cell apoptosis assays of NSCLC cells. (**A**) In vitro experimental procedure; (**B**) viability of the A549 cells and (**C**) the LLC cells upon treatment with XXP in 24 h; depicts (**D**) the visualization and (**E**) outcomes of XXP treatment on the cell cycle in A549 cells; (**F**) illustrates the visualization, and (**G**) presents the findings on cell cycle progression in LLC cells following XXP treatment. (**H**) Images and (**I**) results for apoptosis in A549 cells; (**J**) visualization and (**K**) effect of XXP treatment on apoptosis in LLC cells; (**L**) and (**M**) migration of A549 cells (scale bar: 50 μm); (**N**) and (**O**) migration of LLC cells treated with XXP (scale bar: 100 μm). n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus the control group.

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We found 1550 intersective targets between XXP and lung cancer (Figure 3A) and explored the top 20 differential signaling pathways, including lipid and atherosclerosis metabolism, PI3K/AKT, and mTOR signaling pathways (Figure 3B). CytoNCA analysis identified 19 core targets, including AKT1, ESR1, NR3C1, TP53, and others (Figure 3C). These results offer a foundation for additional exploration into the therapeutic mechanisms of XXP in lung cancer.

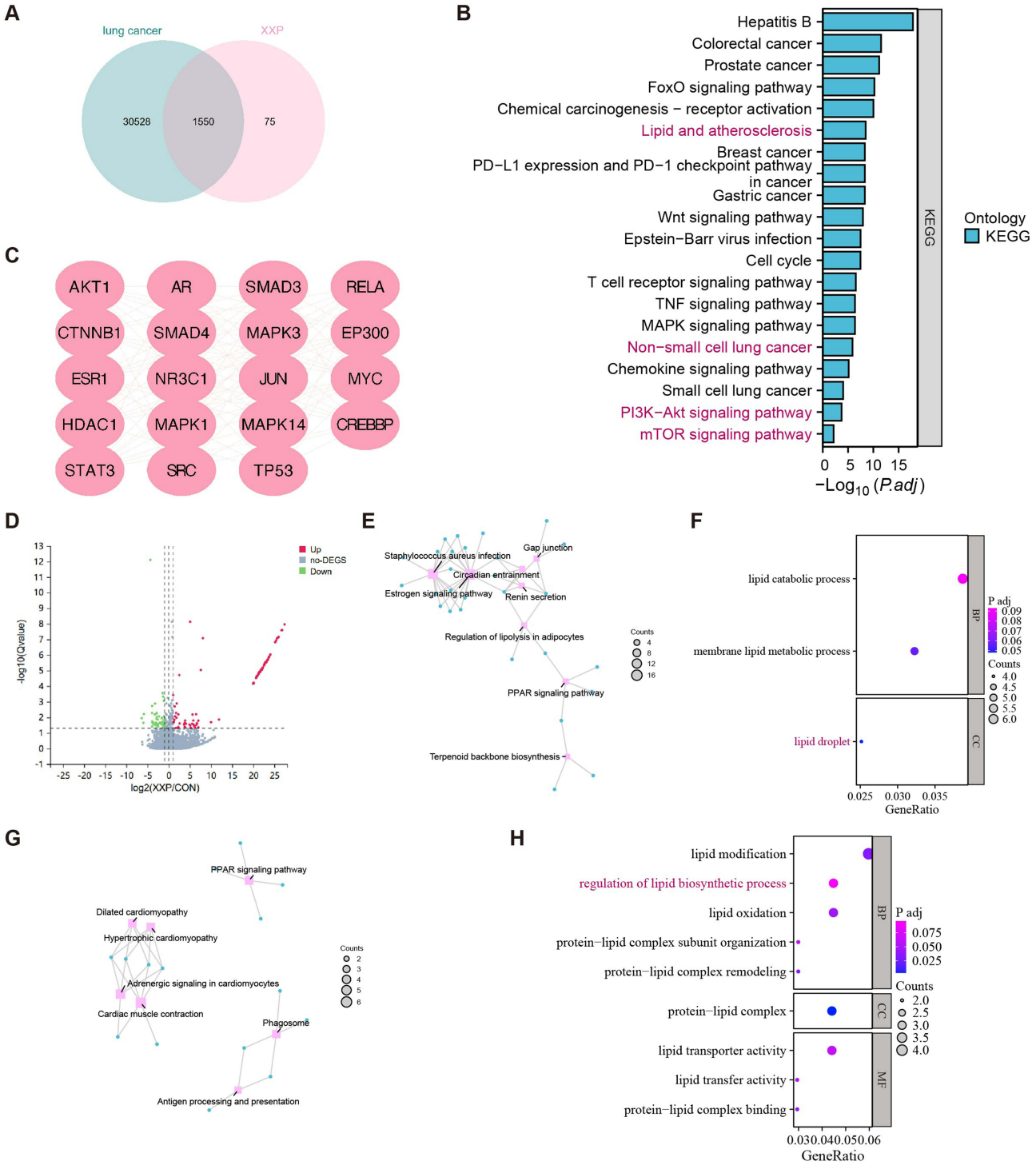

Figure 3 The bioinformatics analysis and the transcriptome sequencing visualization were presented. (**A**) identified the overlapping targets between XXP and lung cancer, (**B**) mapped the KEGG pathways associated with XXP's lung cancer treatment, and (**C**) highlighted the key targets influenced by XXP in lung cancer therapy. (**D**) showed DEGs with red indicating upregulated and green indicating downregulated genes; depicted (**E**) KEGG pathways and (**F**) GO enrichment analysis of upregulated genes in NSCLC treated with XXP; illustrated (**G**) KEGG pathways and (**H**) GO enrichment analysis of downregulated genes in NSCLC treated with XXP.

To confirm the therapeutic mechanism of XXP in NSCLC, we conducted transcriptome sequencing for the H–XXP and MC groups. We detected 237 DEGs and found that 169 DEGs were upregulated and 70 DEGs were downregulated (Figure 3D). KEGG analysis revealed that lipid metabolism appeared for both the upregulated (Figure 3E and F) and downregulated (Figure 3G and H) genes. Consequently, the combined computational and empirical methodology revealed that the modulation of lipid metabolism and the PI3K/AKT/mTOR signaling pathway are pivotal in the therapeutic effects of XXP on NSCLC. Irregularities in lipid metabolism are intricately linked to both the onset and advancement of lung cancer.[31] Lipid metabolism encompasses processes such as fatty acid metabolism, the synthesis of primary bile acids, and the production of steroid compounds.[32]

XXP Regulates Fatty Acid Biosynthesis in Lung Cancer Cells

Fatty acid biosynthesis is a crucial cellular activity that transforms nutrients into metabolic intermediates necessary for the creation of cell membranes, energy reserves, and the production of signaling molecules, thereby facilitating the initiation and progression of tumors.[33] The Oil Red O staining indicated that treatment with XXP at both the IC_{25} and IC_{50} concentrations led to a reduction in lipid droplet accumulation in A549 cells when compared to the control group (Figure 4A and B). Furthermore, at IC_{25} and IC_{50}, XXP reduced free fatty acid contents (Figure 4C). Concentrations of TC and TG were found to be reduced in the groups treated with XXP (IC_{25} and IC_{50}) (Figure 4E and F), suggesting that XXP suppresses the synthesis of fatty acids within NSCLC cells (Figure 4D).

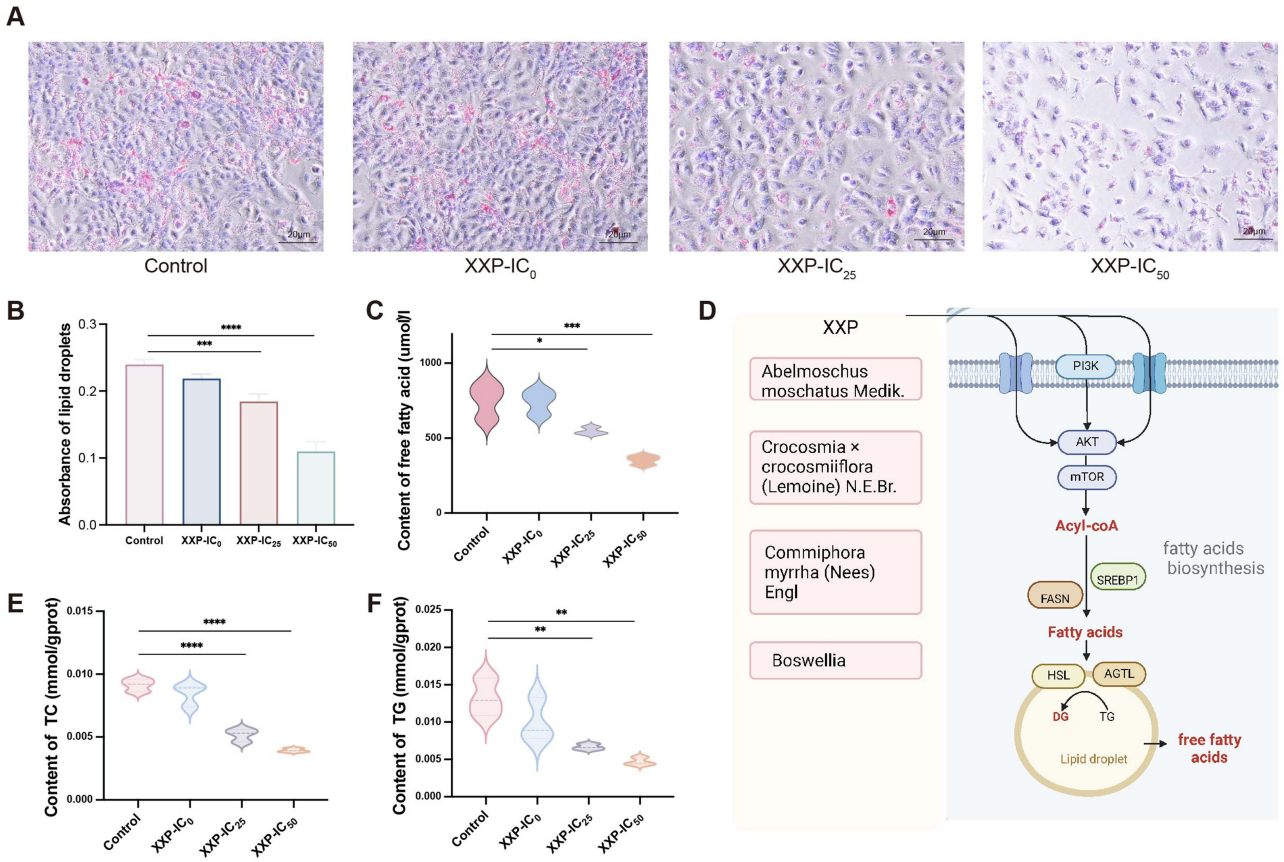

Figure 4 The impact of XXP on fatty acid metabolism. (**A**) and (**B**) displayed Oil Red O staining in A549 cells (scale bar: 20 μm); (**C**) showed contents of the free fatty acids; (**D**) provided a conceptual model of fatty acid metabolism in NSCLC cells. (**E**) TC, and (**F**) TG contents of A549 cells treated by XXP; The experiments were conducted with n = 3, and statistical significance is denoted by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ in comparison to the control group.

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XXP Downregulated PI3K, AKT, FASN, and SREBP1 and Inhibited Fatty Acid Biosynthesis in NSCLC Cells

SREBP1 and FASN are critical transcriptional regulators in fatty acid metabolism. SREBP1, a recently identified transcription factor, is implicated in the regulation of fatty acid synthesis and is observed to be overexpressed in lung carcinoma cells, typically associated with advanced stages and unfavorable prognoses.[34] FASN is reportedly associated with abnormal lipid metabolism in lung cancer.[35] We found XXP downregulated SREBP1 and FASN in A549 cells (Figure 5A–C). The PI3K/AKT/mTOR signaling cascade is pivotal in the modulation of cell growth, metabolism, and tumorigenesis, exerting its influence through the regulation of FASN and SREBP1.[36] In this study, XXP downregulated PI3K, phospho–PI3K, AKT, phospho–AKT, and phospho–mTOR in A549 cells (Figure 5D–L); This potentially contributed to its antitumor effect and its regulatory role in fatty acid metabolism, concurrent with previous reports.[12] Immunofluorescence staining further confirmed XXP decreased expression of phospho–AKT, AKT, SREBP1, and FASN (Figure 5M and N).

XXP Counteracted the Stimulatory Impact of SC79 on the Activation of the PI3K/AKT/mTOR Signaling Axis Within NSCLC Cells

To delve deeper into XXP's ability to counteract the PI3K/AKT signaling pathway's expression in lung cancer, we employed SC79, an AKT-specific activator. Given that AKT is a central component of the PI3K/AKT/mTOR signaling network, the mechanism of its inhibition might be analogous to the one responsible for XXP's dampening effect on the entire PI3K/AKT/mTOR pathway. Our findings revealed that the co-administration of XXP and SC79 led to a decrease in the levels of AKT, phospho–AKT, mTOR, phospho–mTOR, SREBP1 and FASN in comparison to the SC79 group (Figure 6A–G), further elucidating the inhibition expression of XXP on PI3K/AKT/mTOR pathway and effect of fatty acid biosynthesis.

Discussion

XXP is a prototypical prescription drug in TCM, and we found that XXP had a role in improve NSCLC patients' life quality and metabolism in clinical practice. Although the role of XXP in antitumor immunity and in inhibiting tumor angiogenesis has been preliminary proved,[9] XXP's curative effect on NSCLC and its mechanism in fatty acid metabolism have remained unknown.

In our research, we noted that XXP suppressed tumor growth, curbed cell proliferation and migration, triggered apoptosis, regulated the cell cycle, and mitigated the enlargement of tumor nuclei and lymphocyte infiltration both in vivo and in vitro. Our results demonstrated that XXP exerted an inhibitory influence on the progression and development of NSCLC.

The transcriptome sequencing and subsequent bioinformatics analysis revealed that the PI3K/AKT/mTOR signaling pathway and lipid metabolism play roles in the therapeutic effects of XXP on lung cancer. Notably, the metabolism of fatty acids, especially their synthesis, has emerged as a focal point in cancer research, potentially enhancing the early detection, diagnosis, treatment, and prognostic assessment for lung cancer patients.[37]

Fatty acid biosynthesis is crucial for meeting both the energy demands and the extensive biosynthetic needs of tumor cells.[33,38] Dysregulation in this biosynthetic process is a key contributor to the onset of lung cancer. It has been utilized to investigate the disease's progression and to evaluate the effectiveness of therapeutic strategies and prognostic measures.[39] TC, TG, and lipids are outcomes of fatty acid synthesis, reflecting the storage and production of fatty acids within cancer cells.[40] However, fatty acids stored within lipid droplets are catabolized to free fatty acids and glycerol as cancer cells completely expend their energy,[41] thus indirectly representing the extent of fatty acid biosynthesis. We discovered that XXP decreased lipid droplet content, reduced TC and TG levels, and reduced the concentration of free fatty acids. The findings suggest that XXP influences the expression of fatty acid metabolites in NSCLC cells, thereby modulating the fatty acid synthesis process in this type of cancer. Fatty acid biosynthesis is regulated by transcription factors SREBPs and adjusted by key enzyme FASN. SREBP1 is crucial in tumor development and cancer progression, exerting its influence through the regulation of lipid metabolism, immune responses, cell cycle progression, and apoptosis.[42] Its elevated expression in lung cancer tissues is associated with the promotion of lung cancer cell proliferation.[34,43] FASN is

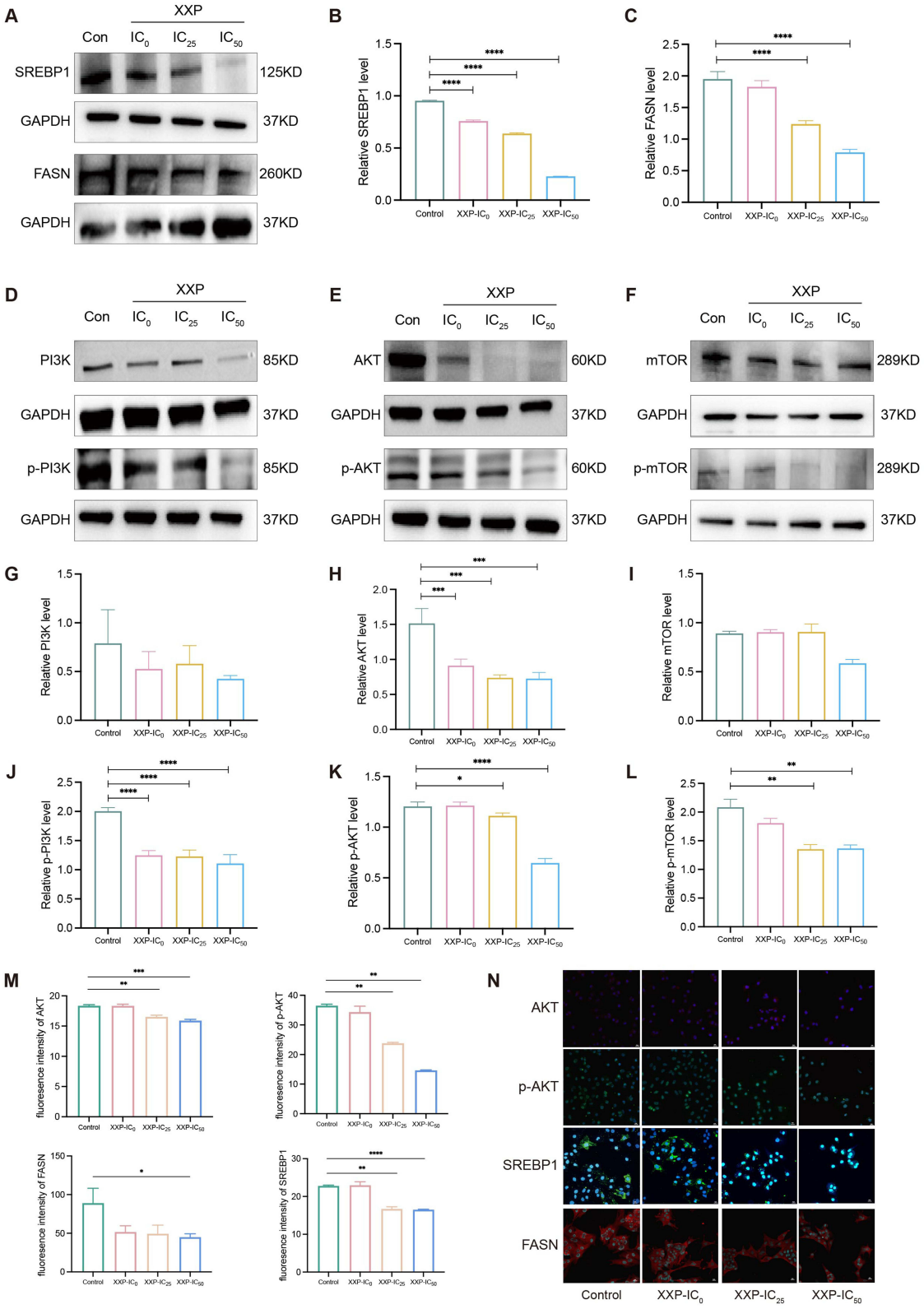

Figure 5 The effect of XXP on the PI3K/AKT/mTOR signaling pathway and fatty acid metabolism in A549 cells. (**A**) Visuals and (**B**) and (**C**) the levels of SREBP1 and FASN proteins involved in fatty acid metabolism in NSCLC cells treated with XXP; (**D**), (**E**), and (**F**) visuals and (**G**), (**H**), (**I**),(**J**), (**K**), and (**L**) the expression levels of PI3K, AKT, mTOR, along with their phosphorylated forms, as well as SREBP1 and FASN, in A549 cells following XXP treatment; (**M**) presented the relative levels of phospho-AKT, AKT, SREBP1, and FASN in A549 cells of immunofluorescence and (**N**) showed the immunofluorescence images. n = 3. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 versus the control group.

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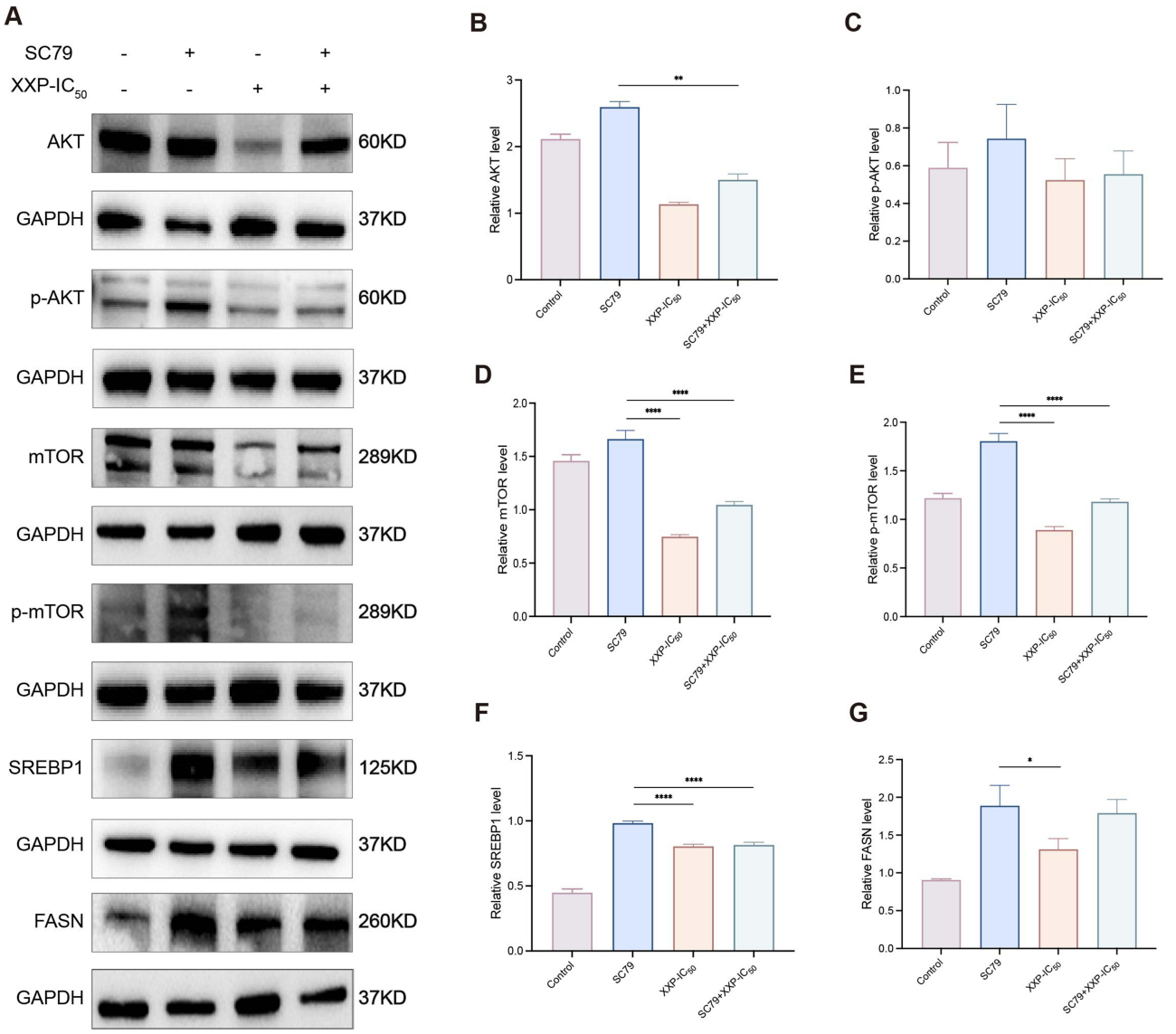

Figure 6 Effects of XXP and SC79 on the PI3K/AKT/mTOR signaling pathway and fatty acid metabolism in A549 cells. (**A**) Results of the protein levels of AKT/mTOR signaling pathway and fatty acid biosynthesis in A549 cells intervened by XXP and SC79, and (**B**–**G**) were the statistic outcomes of them. n = 3. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 versus the SC79 group.

a key enzyme in the synthesis and metabolism of fatty acids, controlling oncogenic signaling and the immunogenicity of tumors.[44] FASN is overexpressed and hyperactivated in several human carcinomas.[45] A study demonstrated that FASN inhibitor exerted a role of anticancer activity to lung cancer.[46] In this study, we observed that XXP could downregulated the expression level of SREBP1 and FASN, thereby highlighting XXP's regulatory impact on these proteins in the context of NSCLC's fatty acid biosynthesis.

The PI3K/AKT/mTOR signaling serves as a pivotal modulator in both cancer progression and the metabolism of fatty acids;[47,48] this pathway regulates the levels of enzymes transcription factors and products associated with fatty acid metabolism.[48] Novel inhibitors of PI3K, AKT, and mTOR have entered clinical trials.[49] We found that XXP down-regulated the PI3K/AKT/mTOR signaling pathway, thus serving as a potential therapeutic inhibitor for NSCLC.

A key advantage of our research is the incorporation of diverse experimental approaches, including in vitro, in vivo, and clinical studies. Nevertheless, a limitation is the absence of direct genetic manipulation to conclusively establish the PI3K/AKT/mTOR pathway's role. Future research employing gene silencing or CRISPR technologies could provide stronger validation of our findings. Additionally, incorporating more NSCLC cell line studies would enhance the robustness of our conclusions.

Conclusion

This research demonstrated that XXP suppressed lung tumorigenesis in vivo and impeded the proliferation, survival, and metastasis of NSCLC cells in vitro. It also targeted the PI3K/AKT/mTOR signaling pathway and fatty acid biosynthesis, indicating a novel therapeutic mechanism. Our findings suggest that XXP could serve as a potential new treatment for NSCLC, acting through the simultaneous inhibition of these key pathways in NSCLC cells.

Abbreviations

AKT, protein kinase B; BATMAN-TCM, bioinformatics analysis tool for molecular mechanism of TCM; DEG, differentially expressed gene; ETCM, encyclopedia of traditional Chinese medicine; FASN, fatty acid synthase; GO, gene ontology; H&E, hematoxylin and eosin; HERB, a high-throughput experiment- and reference-guided database of traditional Chinese medicine; H–XXP, high Xingxiao Pill dose; IC_{25}, 25% inhibitory concentration; IC_{50}, 50% inhibitory concentration; KEGG, Kyoto Encyclopedia of Genes and Genomes; LLC, Lewis lung carcinoma; L–XXP, low Xingxiao Pill dose; MC, model control; mTOR, mammalian target of rapamycin; M–XXP, middle Xingxiao Pill dose; NSCLC, Non-small cell lung cancer; PI3K, phosphoinositide-3-kinase; PPI, protein-protein interaction; SREBP1, sterol regulatory element binding protein 1; TC, total cholesterol; TG, triglyceride; TCM, traditional Chinese medicine; TCMSP, traditional Chinese medicine systems pharmacology database and analysis platform; XXP, Xingxiao Pill.

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Author Contributions

Professor Yanping Bai and Professor Kaiwen Hu serve as co-corresponding authors. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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