Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) *2022 23(8):682-698*
www.jzus.zju.edu.cn; www.springer.com/journal/11585
E-mail: jzus_b@zju.edu.cn

Research Article

https://doi.org/10.1631/jzus.B2200038

Investigation and experimental validation of curcumin-related mechanisms against hepatocellular carcinoma based on network pharmacology

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Abstract: Objective: To determine the potential molecular mechanisms underlying the therapeutic effect of curcumin on hepatocellular carcinoma (HCC) by network pharmacology and experimental in vitro validation. Methods: The predictive targets of curcumin or HCC were collected from several databases. the identified overlapping targets were crossed with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) platform. Two of the candidate pathways were selected to conduct an experimental verification. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) assay was used to determine the effect of curcumin on the viability of HepG2 and LO2 cells. The apoptosis and autophagy of HepG2 cells were respectively detected by flow cytometry and transmission electron microscopy. Besides, western blot and real-time polymerase chain reaction (PCR) were employed to verify the p53 apoptotic pathway and adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) autophagy pathway. HepG2 cells were pretreated with pifithrin-α (PFT-α) and GSK690693 for further investigation. Results: The 167 pathways analyzed by KEGG included apoptosis, autophagy, p53, and AMPK pathways. The GO enrichment analysis demonstrated that curcumin was involved in cellular response to drug, regulation of apoptotic pathway, and so on. The in vitro experiments also confirmed that curcumin can inhibit the growth of HepG2 cells by promoting the apoptosis of p53 pathway and autophagy through the AMPK pathway. Furthermore, the protein and messenger RNA (mRNA) of the two pathways were downregulated in the inhibitor-pretreated group compared with the experimental group. The damage-regulated autophagy modulator (DRAM) in the PFT-α-pretreated group was downregulated, and p62 in the GSK690693-pretreated group was upregulated. Conclusions: Curcumin can treat HCC through the p53 apoptotic pathway and the AMPK/Unc-51-like kinase 1 (ULK1) autophagy pathway, in which the mutual transformation of autophagy and apoptosis may occur through DRAM and p62.

Key words: Curcumin; Network pharmacology; p53; Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK); Apoptosis; Autophagy

1 Introduction

Hepatocellular carcinoma (HCC), the third most fatal cancer type, is one of the major malignant tumors that seriously harm human physical and mental health. The survival time of affected patients is usually only 6–9 months (Brar et al., 2020). It was reported that the abnormal energy metabolism of tumors is one of the ten hallmarks of cancer, and that tumors produce energy mainly utilizing glycolysis, which is known as Warburg effect. This effect in tumors is among the main underlying reasons for their occurrence and development (Fan et al., 2019; Zhu et al., 2020). As a receptor of energy metabolism, studies have shown that the activation of adenosine 5'-monophosphate

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Received Jan. 22, 2022; Revision accepted Apr. 27, 2022;
Crosschecked June 3, 2022

(AMP)-activated protein kinase (AMPK) can effectively inhibit the Warburg effect, counteract the disorder of energy metabolism in tumors, and thus play an anti-tumor role (Vander Heiden et al., 2009). The liver is the primary organ of energy metabolism, and abnormal energy metabolism here is key to the occurrence and development of HCC. Glycolysis is the principal method for producing energy in liver tumor cells, and AMPK can treat HCC by inhibiting glycolysis (Rahib et al., 2014; Liberti and Locasale, 2016). Activated AMPK can promote autophagy by regulating autophagy-related proteins in the mammalian target of rapamycin complex 1 (mTORC1), Unc-51-like kinase 1 (ULK1), and phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3)/vacuolar protein sorting 34 (VPS34) complexes (Li and Chen, 2019; Bai et al., 2022).

Curcumin is a phenolic pigment extracted from the rhizome of *Curcuma* sp. in the family Zingiberaceae. It has been proved to have anti-angiogenesis, anti-inflammatory, antioxidant, anti-tumor, and cell cycle regulatory effects. What is more, it has low toxicity and small side effects on normal cells, making it a potential drug choice for tumor intervention. Studies have verified that curcumin can relieve HCC by inhibiting the growth, proliferation, metastasis, and invasion of liver cancer cells (Zhou et al., 2020) as well as angiogenesis (Chiablaem et al., 2014), and promoting their autophagy (Jiang et al., 2021) and apoptosis (Wang FL et al., 2020). Although the specific mechanism of curcumin in the treatment of HCC has been studied from multiple perspectives, the role of AMPK in the treatment of curcumin on HCC remains to be elucidated.

Network pharmacology was first proposed by Andrew L., who revealed a complex network relationship among disease, gene, target, and drug through interdisciplinary theories such as systems biology, bioinformatics, and multifaceted pharmacology (Hopkins, 2007; Zhang et al., 2019). It constitutes a fast, effective, and economical way to screen therapeutic targets. In our study, network pharmacology was performed to infer the common targets according to the overlapping targets of curcumin and HCC, and then the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was employed to analyze the Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment in core targets. The apoptotic pathway is one of the vital pathways in KEGG analysis. Besides, AMPK-related pathways contain the AMPK/ULK1 autophagy pathway. Studies have revealed that both apoptosis and autophagy can lead to cell death (Wang, 2015). In addition, apoptosis may promote or suppress autophagy and vice versa (Maiuri et al., 2007; Wang, 2015). However, the relationship between apoptosis and autophagy in curcumin-treated HCC remains a mystery. We chose the p53/caspase 9/caspase 3 apoptotic pathway and AMPK/ULK autophagy pathway to further investigate this connection. It was reported that p53/damage-regulated autophagy modulator (DRAM) may induce autophagy (White, 2016; Liu and Liu, 2020), and autophagy may trigger apoptosis through p62 (Moscat and Diaz-Meco, 2009; Islam et al., 2018). Firstly, HepG2 cells were treated with different concentrations of curcumin to explore whether it can treat HCC through apoptosis and autophagy. Then, HepG2 cells were pretreated with pifithrin-α (PFT-α) and GSK690693 to explore the kind of relationship between apoptosis and autophagy in curcumin-treated HCC (Fig. 1).

2 Materials and methods

2.1 Analyses of curcumin and HCC by network pharmacology

2.1.1 Potential targets of curcumin

The possible curcumin targets were collected from four databases: traditional Chinese medicine integrated database (TCMID; http://www.megabionet.org/tcmid) (Xue et al., 2013), the encyclopedia of traditional Chinese medicine (ETCM; http://www.tcmip.cn/ETCM) (Xu et al., 2019), SwissTargetPrediction (http://www.swisstargetprediction.ch) (Gfeller et al., 2013; Daina et al., 2019), and comparative toxicogenomics database (CTD; http://ctdbase.org) (Davis et al., 2019). TCMID is a comprehensive database designed to bridge the gap between traditional Chinese medicine and modern life sciences. ETCM includes comprehensive and standardized information on herbs, formulas of TCM, and their ingredients. SwissTargetPrediction is intended to predict the most probable protein targets of small molecules. Targets with probability>0 were chosen for target screening. CTD can be used to manually curate the scientific literature for chemical-gene, chemical-disease, gene-disease,

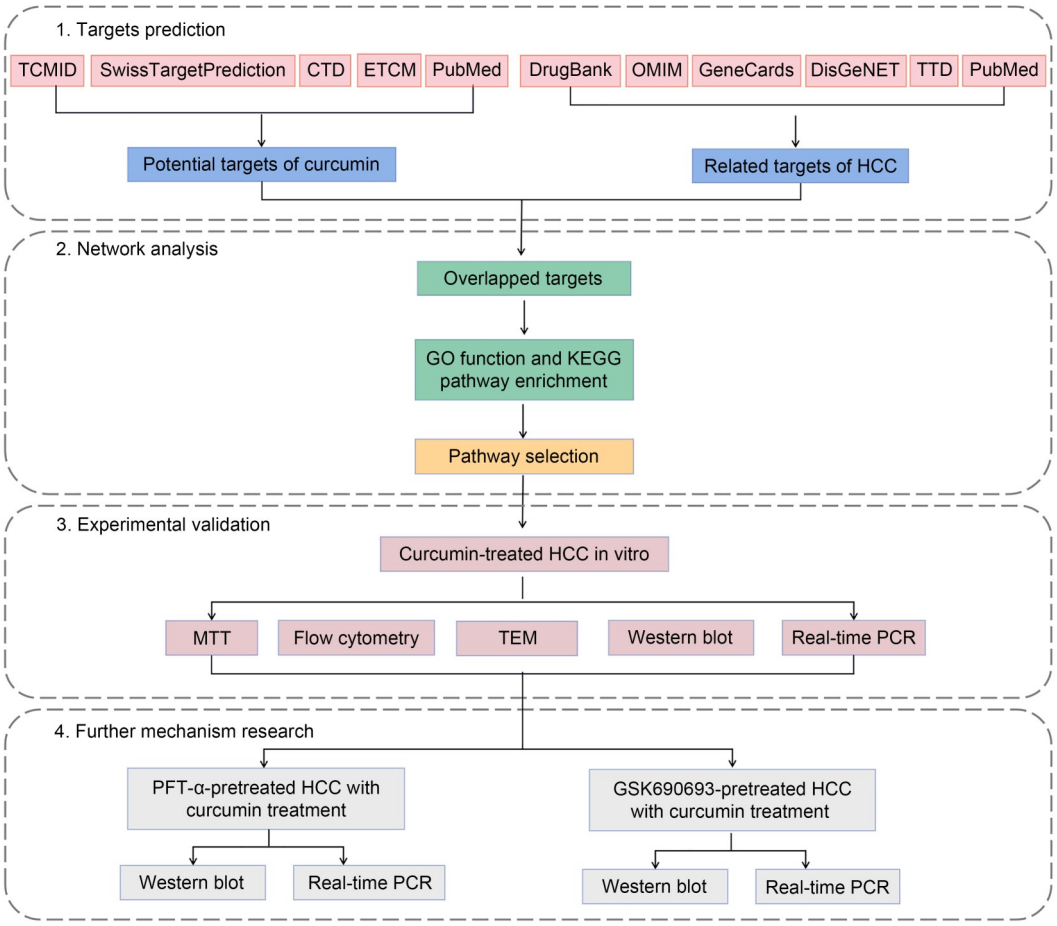

Fig. 1 General framework of this study. TCMID: traditional Chinese medicine integrated database; CTD: comparative toxicogenomics database; ETCM: the encyclopedia of traditional Chinese medicine; OMIM: online mendelian inheritance in man; TTD: therapeutic target database; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium; TEM: transmission electron microscopy; PCR: polymerase chain reaction; PFT-α: pifithrin-α; HCC: hepatocellular carcinoma.

chemical-phenotype, and chemical-exposure associations from all species. The screening criterion was interaction count more than 10. Furthermore, other potential curcumin targets were supplemented by exploring the literature with "curcumin and AMPK" as keywords from the PubMed database (https://pubmed.ncbi.nlm.nih.gov). We picked all targets (gene and protein) in the literature in which curcumin targets contained AMPK. The UniProt (http://www.uniprot.org) was used to convert all therapeutic target names to gene symbols.

2.1.2 Potential targets of HCC

DrugBank-drug and drug target database (https://www.drugbank.ca) (Wishart et al., 2018) is a database containing extensive biochemical and pharmacological information about drugs, their mechanisms, and their targets. Online mendelian inheritance in man (OMIM; https://omim.org) (Amberger et al., 2019) is mainly about human genes and phenotypes and the relationships between them. GeneCards (http://www.genecards.org) (Fishilevich et al., 2017) integrates gene-centric data, including genomic, transcriptomic, proteomic, genetic, clinical, and functional information. We picked out the targets with relevance score of >16.35. Therapeutic target database (TTD; http://db.idrblab.net/ttd) (Wang YX et al., 2020) is a database including the known and explored therapeutic protein and nucleic acid targets, the targeted disease, pathway information, and the corresponding drugs directed at each of these targets. DisGeNET (https://www.disgenet.org) (Piñero et al., 2020) is a comprehensive discovery platform designed to address a variety of questions concerning the genetic underpinning of human diseases.

The screening criterion was of Score_gda>0.3. Then, "hepatocellular carcinoma and AMPK" were used as keywords in the PubMed database to add unrecorded targets. All the genes and proteins of literature curation were picked out as targets, and the HCC-related targets were obtained from these databases. UniProt Knowledgebase (UniprotKB; https://www.uniprot.org/help/uniprotkb) was used to standardize the nomenclature used.

2.1.3 Pathway and biological functional enrichment analysis of overlapped targets

The online website Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny) was used to obtain the intersection of both HCC and curcumin targets and create a Venn diagram. DAVID platform (https://david.ncifcrf.gov) has not only a comprehensive annotation function, but it also updates gene annotation data regularly. The targets of curcumin-treated HCC were recorded into the DAVID platform and the species was set as "Homo Sapiens," and then analyzed by GO function and KEGG pathway enrichment to obtain biological processes and metabolic pathways. These data were saved and R language analysis software was used for visual processing.

2.2 Experimental verification and extension

2.2.1 Chemicals and reagents

Curcumin was purchased from Sigma (St. Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO) to prepare mother liquor at 100 mmol/L. The anti-caspase 3 (19677-1-AP), p53 (60283-2-Ig), and AMPKα (66536-1-Ig) antibodies were obtained from Proteintech Group, Inc. (Wuhan, China). The anti-autophagy-related protein 1 (anti-ATG1)/ULK1 (bs-3602R), phospho-AMPKα2 (Thr172; bs-4002R), phospho-ATG1 (Ser556; bs-3464R), Beclin1 (bs-1353R), microtubule-associated protein light chain 3B (LC3B; bs-4843R), DRAM (bs-10285R), SQSTM1/p62 (bs-55207R), and β-actin (bs-0061R) antibodies were acquired from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The anti-caspase 9 (9508S) antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). The AMPK/ULK1 inhibitor GSK690693 (S1113) and p53 inhibitor PFT-α HBr (S2929) were purchased from Selleck Chemicals (Shanghai, China).

2.2.2 Cell culture and MTT assay

LO2 and HepG2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA; XT-SH30022,01B) supplemented with 12% (volume fraction) fetal bovine serum (Hangzhou Sijiqing, Hangzhou, China; 11011-8611) and 1% (0.01 g/mL) penicillin streptomycin combination (Solarbio, Beijing, China; P1400) at 37 ℃ in a humidified atmosphere containing 5% CO_2, and 0.25% (2.5 g/L) trypsin (Solarbio; T1300) was used for cell passage. To evaluate cell viability, HepG2 or LO2 cells (1×10^4 cells/well) were seeded in 96-well culture plates until the cells attained 80%–90% confluence, and the medium was then replaced with 100 μL/well of fresh medium containing different concentrations (0, 20, 40, and 60 μmol/L) of curcumin for 24 h. Subsequently, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) was added to each well and incubated for 4 h at 37 ℃. The medium was removed and DMSO (150 μL/well) was added, and the plates were shaken on a shaking table for 5–10 min until the dark blue formazan crystals that formed in intact cells were solubilized with DMSO. Finally, the absorbance was measured at 490 nm with a microplate reader.

2.2.3 Flow cytometry assay

In order to evaluate cell apoptosis, HepG2 cells (1×10^5 cells/well) were seeded in six-well culture plates and incubated for 24 h under an atmosphere containing 5% CO_2 at 37 °C. On the second day, the cells were treated with 1 mL of various concentrations (0, 20, 40, and 60 μmol/L) of curcumin. After incubation for 24 h, the cells were washed with cold sterile phosphate-buffered saline (PBS) solution (Boster, CA, USA; PYG0021). Next, the HepG2 cells were resuspended in 100 μL binding buffer (1×10^5 cells) with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Pharmingen, 556507) for 30 min in the dark. After washing with PBS again, cell apoptosis was analyzed using a FACS Caliburt™ Flow Cytometer (BD Biosciences, NJ, USA) within 1 h.

2.2.4 Transmission electron microscopy

HepG2 cells were plated into 6-well plates (1×10^5 cells/well), and cells were divided into groups according to individual cell experiments after HepG2 cells were grown to 80%–90% confluence. The cells

were then digested by trypsin and collected in non-enzymatic EP tubes. After washing with buffer solution, 2.5% (volume fraction) glutaraldehyde buffer stationary solution was added to the cell precipitation overnight. Following further washing with buffer solution, the cells were fixed after adding 2% (volume fraction) osmium tetroxide buffer stationary solution, subjected to sample gradient dehydration with gradient ethanol, and the ethanol in the sample was replaced with acetone. Subsequently, the cells were penetrated with epoxy resin to create an embedded system. Ultra-thin sections of 60 nm were prepared, and the changes of mitochondria, lysosomes, and autophagosomes in the cells were observed under transmission electron microscope (TEM; JEOL, Beijing, China).

2.2.5 Western blot assay

HepG2 cells were treated in six-well culture plates as above. Then, they were washed twice with ice-cold PBS, and harvested in radioimmunoprecipitation (RIPA) lysis buffer containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF) within 1 h on ice. The lysates were centrifuged at 12 000 r/min for 10 min at 4 °C, and the supernatant was collected. According to the results of protein concentration determined by a bicinchoninic acid (BCA) detection kit (Boster; AR0146), protein extracts of equal concentration were prepared with 5× loading buffer, pure water, and the original protein extracts. The samples underwent 8%, 10%, 12%, or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto a polyvinylidene difluoride membrane in a wet transfer system (Bio-Rad, CA, USA). The membrane was blocked with 5% (0.05 g/mL) fat-free milk in Tris-buffered solution containing 1% (volume fraction) Tween 20 (TBST). Next, they were incubated with primary antibodies against AMPKα (1:1000 dilution, volume ratio, the same below), phospho-AMPK (p-AMPK; 1:1000), ULK1 (1:1000), phospho-ULK1 (p-ULK1; 1:1000), LC3B (1:1000), Beclin1 (1:1000), caspase 3 (1:750), caspase 9 (1:1000), p53 (1:300), and β-actin (1:1000) in TBST buffer overnight. On the next day, they were washed and incubated with secondary antibodies for 90 min. Signals were detected using an electrochemiluminescence (ECL) kit (Boster; AR1197), and β-actin served as an internal reference.

Apart from the above treatments, to measure the protein expression of AMPKα, p-AMPK, ULK1, p-ULK1, LC3B, Beclin1, caspase 3, caspase 9, p53, DRAM (1:1000), p62 (1:1000), and β-actin, HepG2 cells were pretreated with 15 μmol/L GSK690693 or 15 μmol/L PFT-α for 2 h, and added into 40 μmol/L of curcumin, according to the above-described method.

2.2.6 Real-time PCR assay

Treated HepG2 cells, different concentrations (0, 20, 40, and 60 μmol/L) of curcumin, 40 μmol/L of curcumin plus 15 μmol/L of GSK690693, and 40 μmol/L of curcumin plus 15 μmol/L of PFT-α were extracted into the non-enzymatic EP tubes. Then, 1 mL of TRIzol universal and 0.2 mL of chloroform were added in sequence. After centrifugation, the top colorless aqueous phase was taken, and 0.5 mL isopropyl alcohol was added. The tube was left at room temperature for 10 min and then centrifuged at 12 000 r/min and 4 °C for 10 min. This was followed by washing the precipitates with 75% (volume fraction) ethanol (diethyl pyrocarbonate (DEPC) water preparation). After drying, they were dissolved in 50 μL of RNase-free distilled water (dH_2O). The concentration and purity of the RNA were determined by the assessment of absorbance at 260 nm and 280 nm using a NanoDrope 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Reverse transcription of RNA was performed into complementary RNA (cDNA) using a PrimeScripte RT Reagent kit (TaKaRa, Beijing, China) according to the manufacturer's instructions. Taking β-actin as an internal reference, the specific primers used for PCR were listed in Table 1.

2.2.7 Statistical analysis

The statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Each experiment was carried out at least three times. Data were presented as mean±standard deviation (SD). The significance of differences was analyzed using one-way analysis of variance (ANOVA), and $P<0.05$ was considered as the threshold.

3 Results

3.1 Potential targets of curcumin

Two protein targets were obtained from the TCMID database, 71 targets were discovered using the SwissTargetPrediction database, 75 targets were

Table 1 Specific primer sequences used in the study

Gene	Upstream primer (5'→3')	Downstream primer (5'→3')
AMPK	CAACTATCGATCTTGCCAAAGG	AACAGGAGAAGAGTCAAGTGAG
ULK1	AACAAGAAGAACCTCGCCAAGTCTC	CCACCGTTGCAGTACTCCATAACC
p62	TGATTGAGTCCCTCTCCCAGATGC	CCGCTCCGATGTCATAGTTCTTGG
p53	GCCCATCCTCACCATCATCACAC	GCACAAACACGCACCTCAAAGC
DRAM	GGTCGTCAGCCGCCTTCATTATC	TCTCTGGAGGTGTTGTTCCCGTATC
β-actin	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC

AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; *ULK1*: Unc-51-like kinase 1; *DRAM*: damage-regulated autophagy modulator.

provided by the CTD database, and 8 targets of curcumin were identified from the ETCM database. A total of 143 drug target proteins were obtained after merging the targets of the four databases. In addition, 121 literature targets were obtained by searching the PubMed database with "curcumin and AMPK" as keywords, and 229 drug targets were finally obtained by eliminating the repeated targets (Table S1).

3.2 Targets related to HCC

The analysis of data from five different databases identified 407 targets associated with HCC. A total of 345 targets were discovered using the GeneCards database, 30 targets were identified by the DrugBank database, 64 targets were obtained from the OMIM database, 29 targets were provided by the TTD database, and 9 targets were found in the DisGeNET database. What is more, the PubMed database was used to find 63 targets with "hepatocellular carcinoma and AMPK" as keywords, and 445 disease targets were ultimately obtained (Table S2).

3.3 Curcumin-target-HCC network analysis

The intersections of screened curcumin targets and HCC targets were obtained by Venny 2.1, and a Venn diagram was drawn. As shown in Fig. 2a, 102 common targets of curcumin in HCC were acquired, which included the intersection targets of AMPK and p53 (Table S3). GO and KEGG enrichment analyses were performed on the corresponding targets of curcumin through the DAVID platform. The KEGG enrichment analysis revealed 167 pathways of curcumin-treated HCC (Table S4), including the PI3K-Akt pathway, hepatitis B, human papillomavirus infection, proteoglycans in cancer, apoptosis, autophagy, p53 pathway (Fig. 2b), and AMPK pathway (Fig. 2c). The first 20 passages of the KEGG pathway enrichment analysis were imported into R language analysis software for visual processing (Fig. 2d). The GO enrichment analysis showed that curcumin had a total of 2405 biological processes, such as cellular response to chemical stress, cellular response to drug, regulation of apoptotic pathways, and reactive oxygen species metabolic process. One hundred and fifty molecular features were detected to regulate HCC, containing phosphatase binding, protein serine/threonine kinase activity, protein phosphatase binding, cytokine activity, etc. Besides, we found 41 HCC cell components, including cyclin-dependent protein kinase holoenzyme complex, transcription regulator complex, etc. The specific GO analysis results were shown in Table S5 and Fig. 2e.

3.4 Inhibition of HepG2 cell proliferation by curcumin

In order to study the inhibitory effect of curcumin on hepatoma cells and normal liver cells, MTT assay was used to detect cell viability in vitro. The results showed that curcumin inhibited the activity of LO2 cells and HepG2 cells in a dose-dependent manner; however, with the increase of drug dose, the inhibitory rate of LO2 cells became significantly lower than that of HepG2 cells, especially at a high dose of 60 μmol/L (P<0.01; Fig. 3a), indicating that curcumin caused little damage to normal liver cells and exerted an obvious inhibitory effect on the proliferation of hepatoma cells.

3.5 Effects of curcumin on HepG2 cell apoptosis and autophagy

Our group used Annexin V-FITC/PI staining to detect the apoptosis rate of HepG2 cells, and the results demonstrated that curcumin at the concentration of 20, 40, and 60 μmol/L significantly induced the apoptosis of HepG2 cells compared with the control group (P<0.01; Figs. 3b and 3c). Furthermore, autophagosomes and lysosomes were found by TEM in the experimental group, indicating the occurrence of autophagy (Fig. 3d).

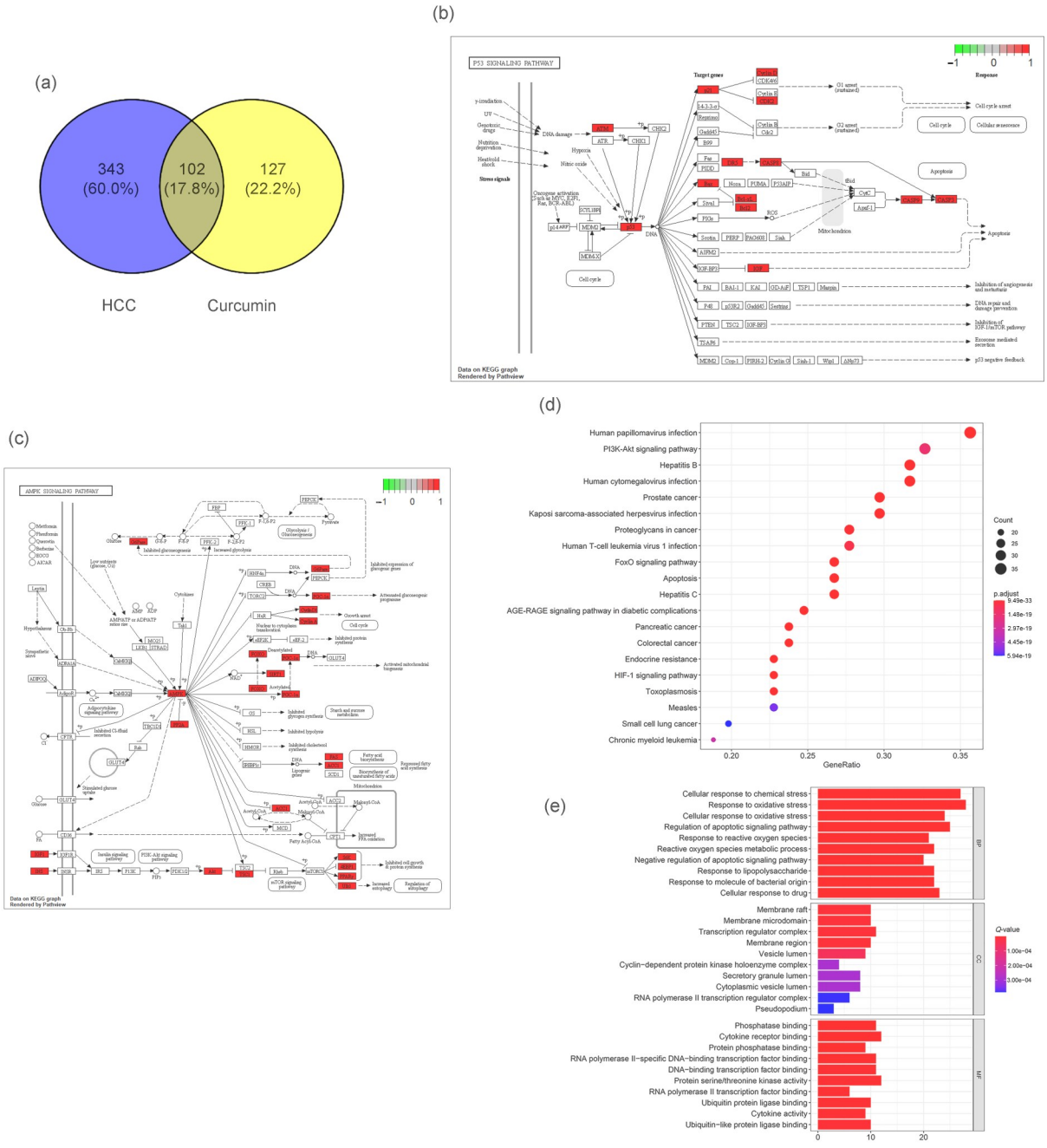

Fig. 2 Network pharmacology on curcumin and hepatocellular carcinoma (HCC). (a) Analysis of HCC and curcumin targets by Venny 2.1. (b) p53 pathway enrichment genes *p53*, *caspase 9*, and *caspase 3* are related to apoptosis. (c) AMPK pathway enrichment genes *AMPK* and *ULK1* are related to autophagy. (d) KEGG analysis bubble diagram of curcumin against HCC targets. (e) GO enrichment analysis of curcumin targets for HCC treatment. AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; ULK1: Unc-51-like kinase 1; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; BP: biological process; CC: cell component; MF: molecular feature.

3.6 Promotion of HepG2 cell apoptosis by curcumin through the p53 pathway

Previous network pharmacology demonstrated that curcumin-treated of HCC was closely related to apoptosis, and the p53 pathway was also linked to apoptosis. Given that apoptosis plays an important role in tumor cell death, we speculated that the curcumin-dependent p53 pathway may be partly induced by the initiation of apoptosis. To examine

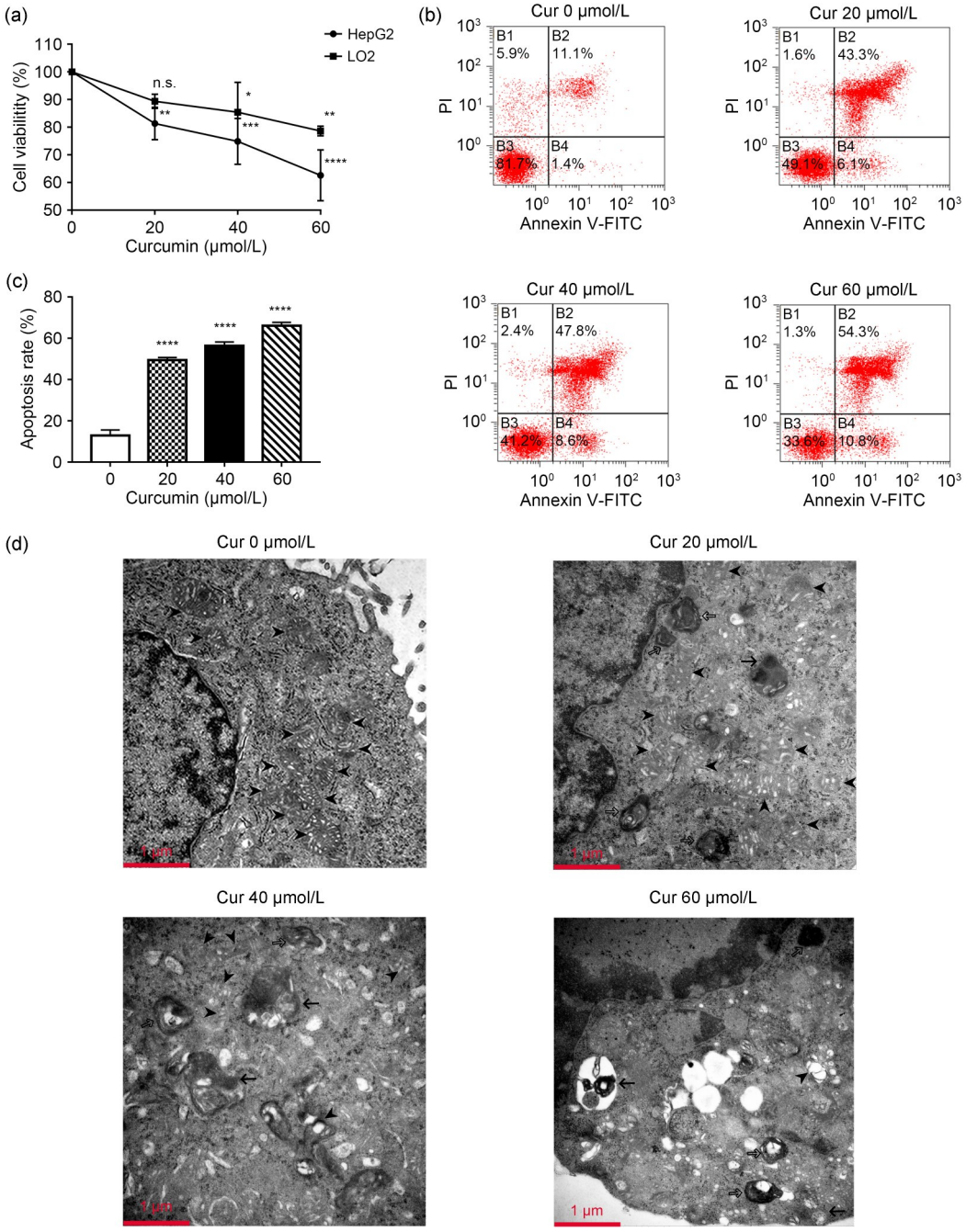

Fig. 3 Effects of curcumin on HepG2 cell proliferation, cell apoptosis, and autophagy. Cell experiments were divided into four groups: control group (no curcumin); curcumin (20 μmol/L) group; curcumin (40 μmol/L) group; and curcumin (60 μmol/L) group. Data were expressed as mean±standard deviation (SD), n=3. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the control group. (a) Cell survivals of HepG2 and LO2 cells. (b, c) Representative plots and quantification of apoptosis rates of HepG2 cells. (d) The changes of mitochondria, lysosomes, and autophagosomes in the cells under transmission electron microscope (TEM; 30000×). The arrowhead marks mitochondria, the single arrow marks autophagosome, and the hollow arrow marks lysosome. Cur: curcumin; FITC: fluorescein isothiocyanate; PI: propidium iodide.

curcumin-induced apoptosis, the three apoptosis proteins, p53, caspase 3, and caspase 9, were analyzed by western blot. Under the stimulation of curcumin with different concentrations for 24 h, it could upregulate the protein expression of p53, caspase 3, and caspase 9 in a dose-dependent manner (Figs. 4a–4d).

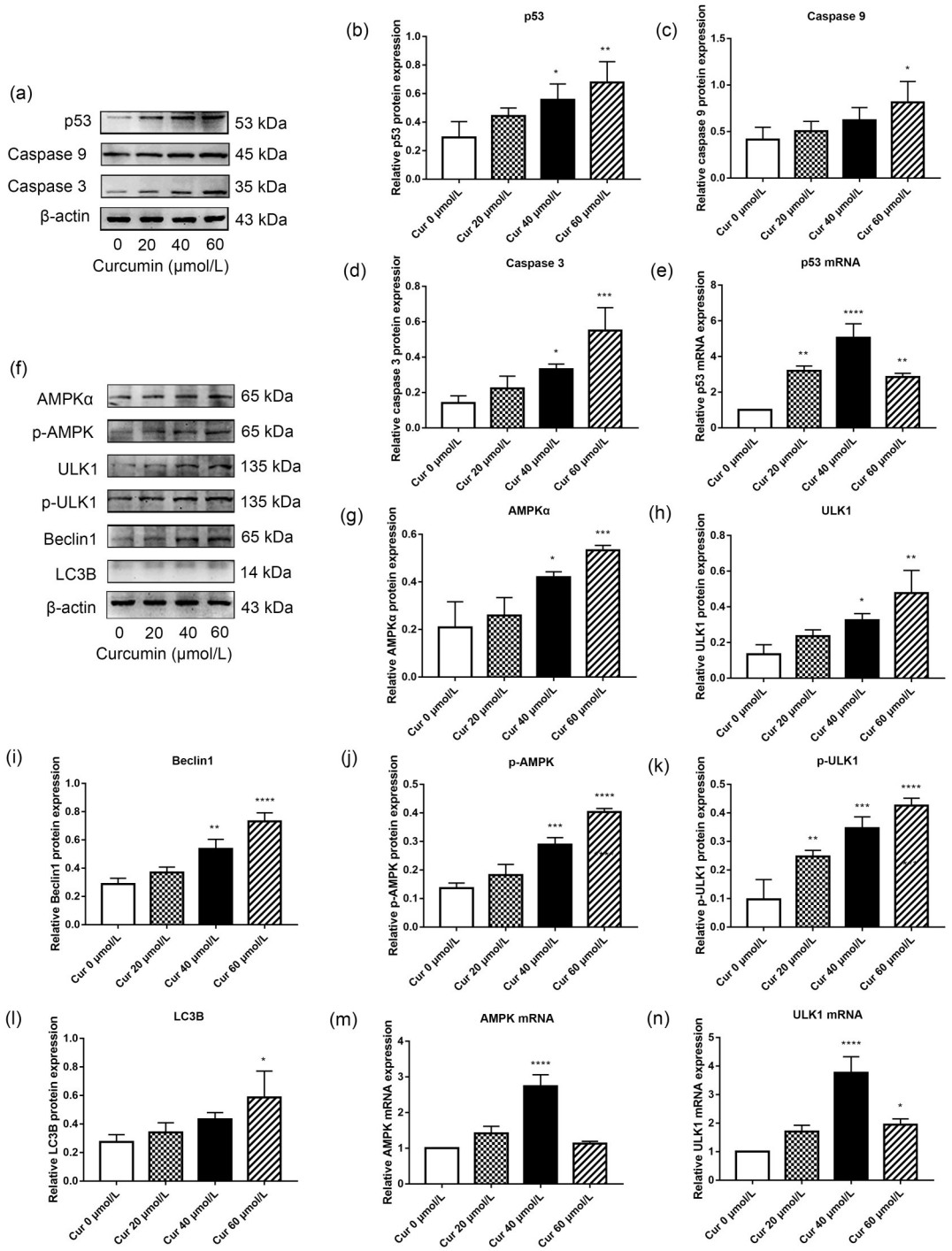

Fig. 4 Curcumin-induced apoptosis and autophagy of HepG2 cells through the p53 and AMPK/ULK1 pathway, respectively. HepG2 cells were treated with different concentrations of curcumin (0, 20, 40, and 60 μmol/L) for 24 h. The data are shown as mean±standard deviation (SD), n=3. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the control group. (a–d) The protein expression levels of p53, caspase 3, and caspase 9 were determined after curcumin treatment. (e) The messenger RNA (mRNA) expression of p53. (f–l) The cells were collected and subjected to western blot analysis, with indicated antibodies against the AMPK/ULK1 pathway associated with molecules AMPKα, phospho-AMPK (p-AMPK), ULK1, phospho-ULK1 (p-ULK1), Beclin1, and LC3B. (m, n) Real-time polymerase chain reaction (PCR) analyses of AMPK and ULK1 mRNA expression levels. AMPKα: adenosine 5'-monophosphate (AMP)-activated protein kinase α; ULK1: Unc-51-like kinase 1; LC3B: microtubule-associated protein light chain 3B; Cur: curcumin.

The messenger RNA (mRNA) expression of p53 was significantly upregulated (Fig. 4d). What is more, with the increase of curcumin concentration, TEM revealed that the mitochondria swelled, and the number of mitochondrial ridges decreased, which were disordered and even disappeared, eventually forming vacuolar mitochondria, indicating that curcumin could cause mitochondrial damage in HepG2 cells (Fig. 3d). These results suggested that curcumin plays an anticancer role in HepG2 cells through p53, caspase 9, and caspase 3 pathways, and regulates the hepatoma cell survival of curcumin through apoptosis mediated by mitochondria.

3.7 Promotion of the autophagy of HepG2 cells by curcumin through the AMPK/ULK1 pathway

According to the literature and network pharmacology, there are autophagy and AMPK pathways in curcumin-treated HCC, and the AMPK/ULK1 pathway can promote the autophagy of HCC. The western blot results of the predicted targets AMPKα and ULK1 showed that curcumin could upregulate their protein expression in a dose-dependent manner (Figs. 4f–4h). The protein expression levels of two autophagy biomarkers, LC3B and Beclin1, were increased, and the protein expression levels of p-AMPK and p-ULK1 were upregulated (Figs. 4f, 4i–4l). Moreover, the mRNA expression levels of AMPK and ULK1 were also significantly upregulated (Figs. 4m and 4n). All the above results suggested that curcumin played an anticancer role in HepG2 cells through the AMPK/ULK1 pathway, and mainly regulated the effect on hepatoma cell survival through autophagy.

3.8 Effect of PFT-α on the apoptosis and autophagy of liver cancer cells induced by curcumin

In order to verify the role of curcumin in PFT-α-pretreated HepG2 cell vitality in vitro, PFT-α was used, which is a p53 inhibitor that blocks p53-dependent p53 response gene transcription. HepG2 cells were pretreated with 15 μmol/L of PFT-α for 2 h, and then 40 μmol/L of curcumin was added for 24 h. Compared with the experimental group (40 μmol/L of curcumin), both the protein and the mRNA expression levels of p53 apoptotic pathway were obviously downregulated in the PFT-α-pretreated group (Figs. 5a–5e). Furthermore, the protein and mRNA expression levels of AMPK/ULK1 pathway and autophagy biomarkers were also significantly decreased (Figs. 5f–5l). These results suggested that PFT-α-pretreated HepG2 cells with curcumin treatment could inhibit not only the p53 apoptotic pathway, but also the AMPK/ULK1 autophagy pathway.

3.9 Disruption of autophagy and apoptosis by GSK690693 of liver cancer cells induced by curcumin

GSK690693 is an autophagy inhibitor that can effectively block AMPK and death-associated protein kinase 3 (DAPK3) of the calcium/calmodulin-dependent protein kinase (CAMK) family. Meanwhile, GSK690693 can affect the activity of ULK1 and effectively inhibit the activation of STING-dependent interferon regulatory factor 3 (IRF3). As shown in Fig. 6, our study found that the proteins of AMPK/ULK1 pathway and autophagy biomarkers were markedly downregulated in the GSK690693-pretreated group as compared with the experimental group. Moreover, the mRNA expression levels of both AMPK and ULK1 were decreased to the same extent. In addition, the p53 protein and mRNA expression levels and their apoptotic pathway protein expression levels were significantly downregulated (P<0.05; Fig. 6). The overall results above indicated that AMPK/ULK1 inhibitors could not only inhibit the AMPK/ULK1 autophagy pathway, but also the p53 apoptotic pathway.

3.10 Mutual transformation between apoptosis and autophagy on curcumin-treated hepatoma cells

DRAM is a lysosomal protein encoded by the p53 target gene, which can induce macroautophagy, and is also a medium for programmed cell death induced by p53 (Crighton et al., 2006). The western blot and real-time PCR analysis showed that DRAM was downregulated in the PFT-α-pretreated group compared with the experimental group (P<0.05; Figs. 7a–7c). Studies have confirmed that the loss of p62 ZZ domain can restore the apoptosis sensitivity of cells in tumors, and thus autophagic apoptosis is recognized to be involved in p62 (Moscat and Diaz-Meco, 2009; Lee et al., 2021). As shown in Figs. 7d–7f, the expression of p62 protein and mRNA suggested that p62 levels in the pretreatment groups of GSK690693 were both upregulated when compared with that in the experimental group (P<0.05).

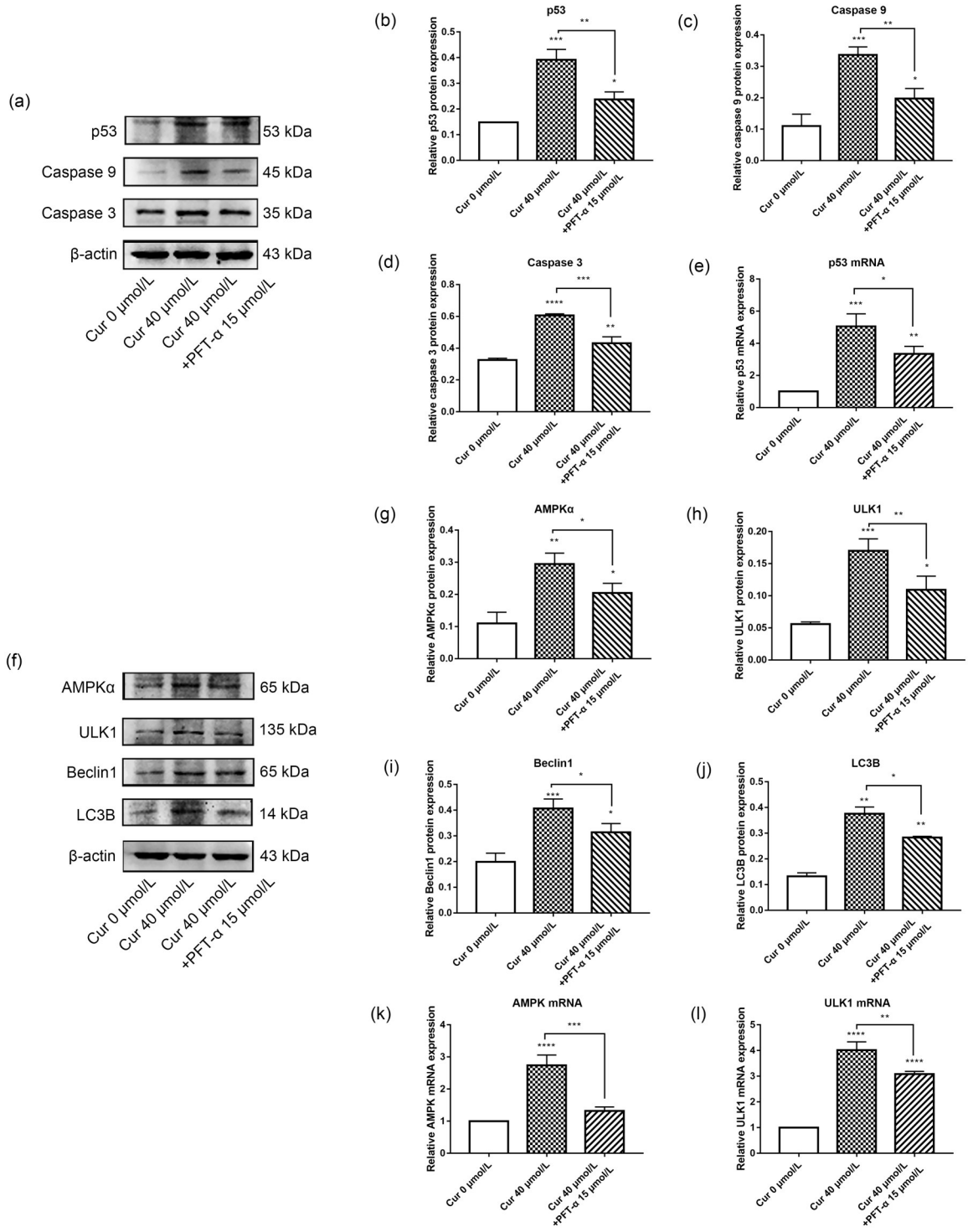

Fig. 5 Effect of p53 inhibitor PFT-α on the curcumin-induced apoptosis and autophagy of hepatoma cells. The data are shown as mean±standard deviation (SD) (n=3). * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the control group or the PFT-α-pretreated group. (a–d) Western blot images and quantitative analysis of p53 apoptotic signal pathways. (e) Real-time polymerase chain reaction (PCR) analysis of p53 messenger RNA (mRNA) expression. (f–j) Western blot images and quantitative analyses of the protein expression levels of autophagy biomarkers (AMPKα, ULK1, Beclin1, and LC3B). (k, l) Real-time polymerase chain reaction (PCR) analyses of AMPK and ULK1 mRNA expression levels. PFT-α: pifithrin-α; AMPKα: adenosine 5'-monophosphate (AMP)-activated protein kinase alpha; ULK1: Unc-51-like kinase 1; LC3B: microtubule-associated protein light chain 3B; Cur: curcumin.

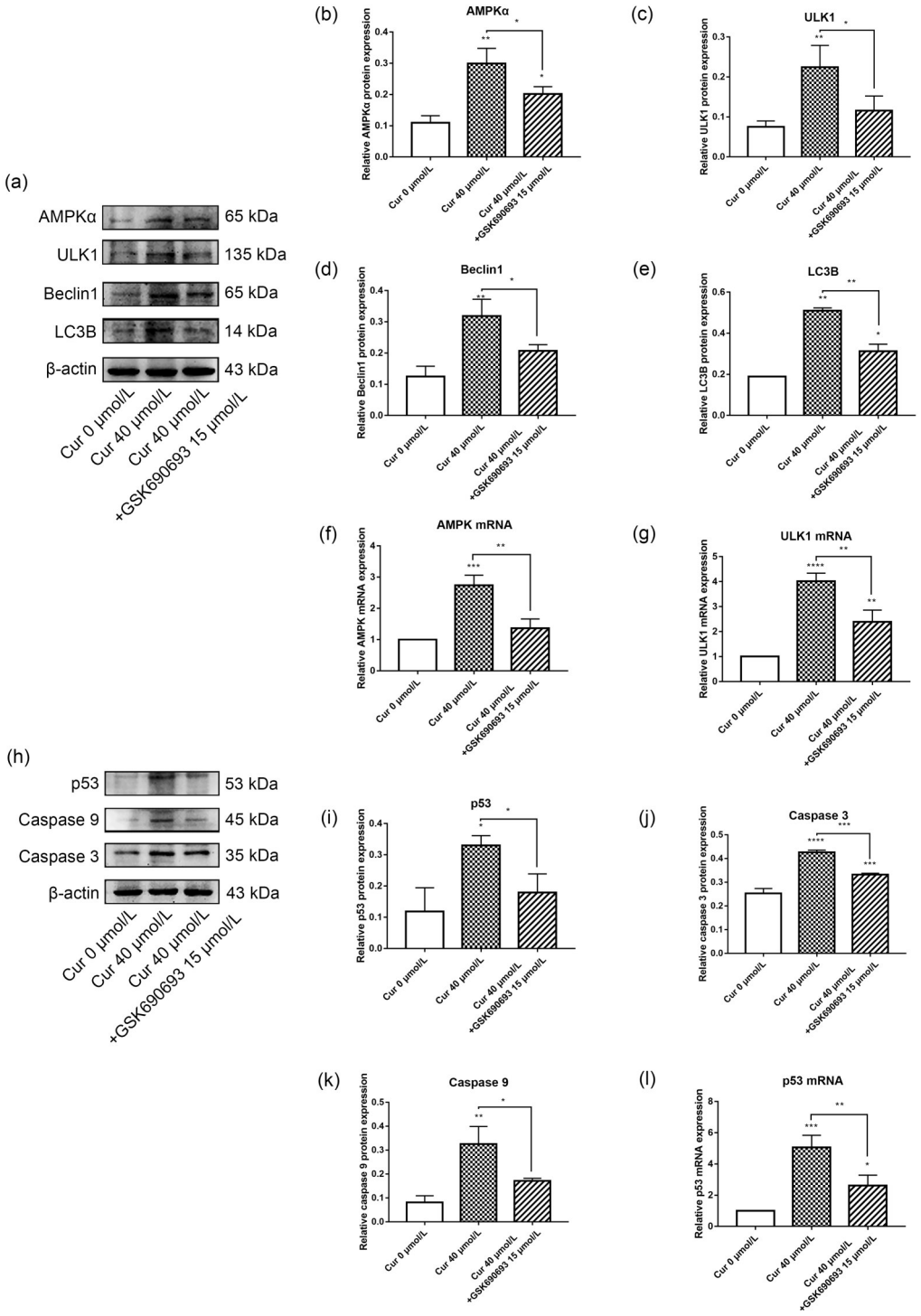

Fig. 6 Effects of GSK690693 inhibitor on curcumin-induced autophagy and apoptosis of hepatoma cells. The data are shown as mean±standard deviation (SD), n=3. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the control group or the GSK690693-pretreated group. (a–e) Western blot images and quantitative analyses of the protein expression levels of autophagy pathway and autophagy biomarkers (AMPKα, ULK1, Beclin1, and LC3B). (f, g) Real-time polymerase chain reaction (PCR) analyses of AMPK and ULK1 messenger RNA (mRNA) expression. (h–k) Western blot images and quantitative analyses of p53 apoptotic pathways. (l) Real-time PCR analysis of p53 mRNA expression. AMPKα: adenosine 5'-monophosphate (AMP)-activated protein kinase α; ULK1: Unc-51-like kinase 1; LC3B: microtubule-associated protein light chain 3B; Cur: curcumin.

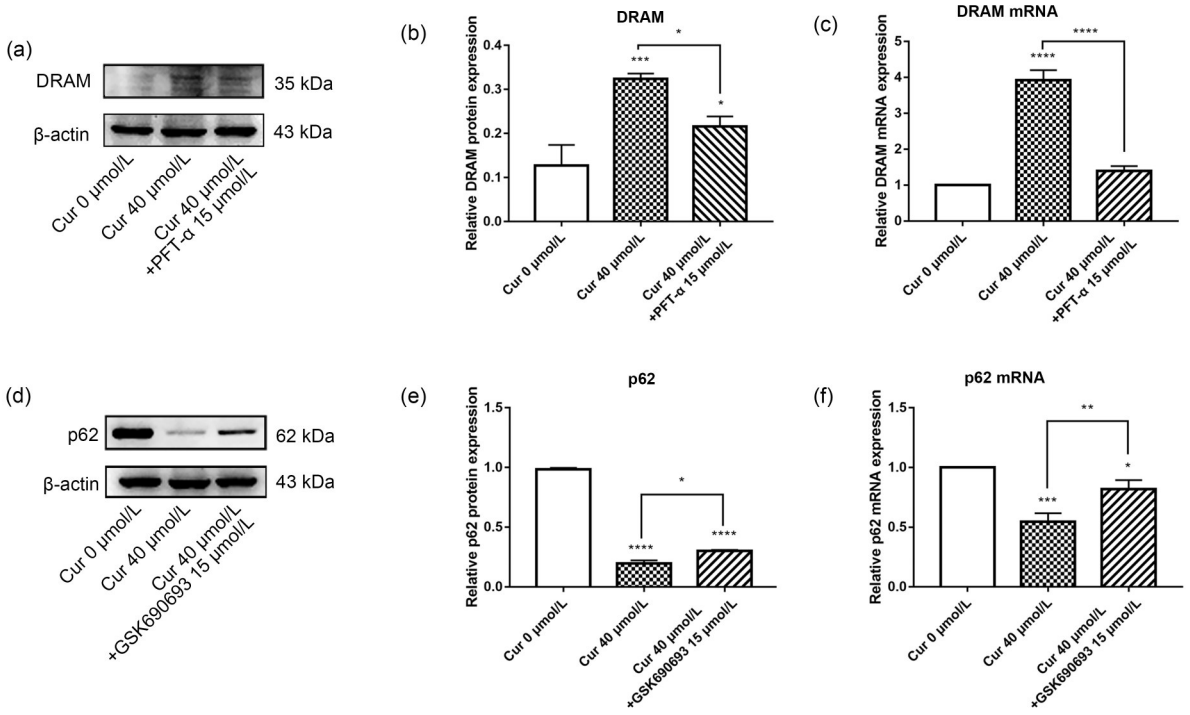

Fig. 7 Inhibition of curcumin-induced autophagy by PFT-α pretreatment of HepG2 cells through DRAM, and inhibition of curcumin-induced apoptosis by GSK690693 pretreatment of HepG2 cells through p62. The data are shown as mean± standard deviation (SD), n=3. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, compared with the control group, the PFT-α-pretreated group, or the GSK690693-pretreated group. (a–c) Western blot and real-time polymerase chain reaction (PCR) analyses of DRAM. (d–f) Western blot and real-time PCR analyses of p62. PFT-α: pifithrin-α; DRAM: damage-regulated autophagy modulator; Cur: curcumin.

4 Discussion

HCC is a highly prevalent and fatal tumor. At present, conventional chemotherapy, liver transplantation, radiofrequency ablation, and arterial revascularization are commonly used in the clinical treatment of HCC. However, due to its frequent recurrence and low objective response rate, the therapeutic effect is often unsatisfactory (Li et al., 2016). It is well known that liver is the main site of energy metabolism in the body, and AMPK is the principal sensor of energy metabolism. Glycolysis is the fundamental process for liver tumor cells to produce energy. AMPK can treat HCC by inhibiting glycolysis, i.e., the Warburg effect (Hardie and Alessi, 2013). Donadon et al. (2010) found that the long-term use of metformin (AMPK activator) could significantly reduce the incidence of HCC in clinical settings, and Zheng et al. (2013) reported that the inhibition of AMPK activation could promote the progression of HCC. Curcumin has been confirmed as a potential anti-liver cancer drug (Darvesh et al., 2012; Ailioaie and Litscher, 2020). In our study, 102 common targets and 167 pathways of curcumin and HCC were established by network pharmacology. The KEGG enrichment analysis demonstrated that apoptotic pathway was one of the first ten passages, and that caspase 9 and caspase 3 in the p53 pathway could promote apoptosis. Besides, the AMPK/ULK1 autophagy pathway was one of the related AMPK pathways. To the best of our knowledge, curcumin in the treatment of HCC may affect not only the apoptotic signaling molecules, including p53, caspase 9, and caspase 3 (Liu et al., 2017), but also the autophagic signaling molecules, including LC3B, Beclin1, and cytochrome *c* (Glick et al., 2010; Onorati et al., 2018; Jiang et al., 2021). However, no previous studies on the treatment of HCC by curcumin have confirmed the role of AMPK/ULK1 autophagy pathway. Our experimental results indicated that curcumin could promote cell apoptosis through the p53 pathway and promote autophagy through the AMPK/ULK1 pathway, thus reducing the survival rate of HepG2 hepatoma cells to achieve

the purpose of HCC treatment, which was consistent with the prediction results of network pharmacology.

As widely known, apoptosis, which represents type I programmed cell death, is an active cell death process regulated by genes during ontogeny (D'Arcy, 2019). The important mechanism of tumor escape is immune-controlled cell apoptosis. Autophagy or type II programmed cell death is vital for cell self-digestion, degradation, and homeostasis maintenance (D'Arcy, 2019). It has been proved that autophagy plays a complex bidirectional regulatory role in tumors. At the early stage of tumor occurrence, autophagy can remove damaged organelles of senescent cells, thus preventing chromosomal variation and inhibiting the initiation of tumorigenesis (Liang and Jung, 2010). In the process of tumor growth, tumor cells obtain energy and nutrients to maintain their growth and reproduction after autophagy degradation and reabsorption (Quan and Lee, 2013). Studies have revealed that autophagy and apoptosis have two relationships, that is, mutual promotion and mutual antagonism. When the ultimate goal of autophagy and apoptosis is to promote cell death, there are three kinds of relationships between these two phenomena. The first is that they regulate cell death separately; the second is a mutual complementary relationship (i.e., the two can be transformed into each other); the third is a substitution relationship (i.e., if one is weakened, the other is strengthened) (Maiuri et al., 2007; Mariño et al., 2014; Wang, 2015). Previous studies have demonstrated that curcumin could alleviate HCC by inducing apoptosis and autophagy (Xu et al., 2013; Jiang et al., 2021), but the relationship between these two processes is unknown. In this study, the pretreatment of HepG2 cells with PFT-α or GSK690693 and then their treatment with 40 μmol/L of curcumin demonstrated that either PFT-α or GSK690693 could inhibit cell apoptosis and autophagy, which suggested that there may be a second relationship between apoptosis and autophagy in curcumin-treated HCC.

Studies have confirmed that p53 in the nucleus can activate autophagy through a variety of pathways, including the activation of AMPK and then the activation of tuberous sclerosis complex 1 (TSC1)/TSC2 complex to promote autophagy, upregulation of DRAM to induce autophagy, etc. (Chen and Debnath, 2010; Jing et al., 2011). DRAM was reported as the first directly linked molecule of p53 and autophagy; apart from DRAM-induced autophagy, DRAM is also of importance in p53-induced apoptosis (Crighton et al., 2006, 2007; Liu and Liu, 2020). Our study showed that DRAM in the PFT-α-pretreated group was lower than that in the experimental group, suggesting that autophagy induced by the p53 apoptosis protein may occur through the increase of DRAM expression in curcumin-treated HCC. In recent years, research highlighted that the p73 of p53 family could also induce autophagy, whose mechanism might be related to the activation of ATG5 (He et al., 2013). Besides, p53 can upregulate autophagy by inhibiting mTORC1 (Chen and Debnath, 2010). The above relationship possibly exists, although it needs more specific evidence. The p62 protein is involved in the autophagy degradation of ubiquitinated proteins, but multiple studies have considered p62 as a molecule linking autophagy and apoptosis (Jin et al., 2009; Katsuragi et al., 2015; Lamark et al., 2017). In tumors, p62 reduction could restore cell sensitivity to apoptosis; p62 in the AMPK/ULK1 inhibitor GSK690693-pretreated group was higher than that in the curcumin-treated group, which indicated that autophagy-induced apoptosis might be related to p62 reduction. Zhao et al. (2020) found that oroxylin A triggered apoptosis through activating caspase 8 and hydrolyzing p62 in HCC cells, which might be a p62-mediated caspase 8-dependent form of apoptosis. The specific mechanism may be that caspases are recruited into the autophagosome that acts as an intracellular activation platform. In the process of being recruited to an autophagosome, ubiquitin caspase 8 binds to p62 through the ubiquitin-binding domains of p62, and then p62 directly interacts with LC3 to be recruited to an autophagosome. Importantly, p62 not only enforces caspase 8 into autophagosomes but also activates caspase 8 (Julien and Wells, 2017; Tsapras and Nezis, 2017).

5 Conclusions

Despite the potential of the network pharmacology approach, some targets and pathways have not been reported. Herein, we used this tool to demonstrate that curcumin may inhibit the proliferation and survival of HCC via apoptosis through the p53 pathway and autophagy through the AMPK pathway. Our findings

further suggested that there may be a mutual transformation of autophagy and apoptosis to promote cell death, which occurs through DRAM and p62 in HepG2 cells.

Acknowledgments

This work was supported by the General Project of Shaanxi Science and Technology Plan (No. 2021JM-472), the Key Laboratory Project of Education Department of Shaanxi Province (Nos. 21JS014 and 21JS007), the Subject Innovation Team of Shaanxi University of Chinese Medicine (No. 2019-YL14), and the Postgraduate Student's Innovation Project of Shaanxi University of Chinese Medicine (No. 2021-09), China.

Author contributions

Yang CHEN, Yu FAN, and Dongyan GUO designed the experiments and analyzed the data. Yang CHEN, Qian LI, and Sisi REN performed the experimental research. Ting CHEN, Bingtao ZHAI, and Jiangxue CHENG analyzed the experimental data. Bingtao ZHAI and Dongyan GUO verified the experiments. Xiaoyan SHI and Liang SONG guided the experiment. Yang CHEN wrote and edited the manuscript. Yu FAN and Dongyan GUO revised this manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yang CHEN, Qian LI, Sisi REN, Ting CHEN, Bingtao ZHAI, Jiangxue CHENG, Xiaoyan SHI, Liang SONG, Yu FAN, and Dongyan GUO declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Ethical approval was not required in accordance with local guidelines.

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Supplementary information

Tables S1–S5