PHARMACEUTICAL BIOLOGY
2021, VOL. 60, NO. 1, 75–86
https://doi.org/10.1080/13880209.2021.2017468

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Deciphering the molecular mechanism of tetrandrine in inhibiting hepatocellular carcinoma and increasing sorafenib sensitivity by combining network pharmacology and experimental evaluation

Biao Niu, Sidong Wei, Jianjun Sun, Huibo Zhao, Bing Wang and Guoyong Chen

Department of Hepatobiliary and Pancreas Surgery, Henan Provincial People's Hospital, Zhengzhou, China

ABSTRACT

Context: The mechanism of tetrandrine (TET) in hepatocellular carcinoma (HCC) progression and sorafenib (Sora) chemosensitivity deserves investigation.

Objective: Using network pharmacology approaches to elucidate the mechanisms of TET in HCC.

Materials and methods: CCK-8, colony formation, and flow cytometry assays were used to measure cell phenotypes. BALB/c nude mice were divided into Control, Sora (10 mg/kg), TET (50 mg/kg), and TET + Sora (10 mg/kg Sora plus 50 mg/kg TET) groups to evaluate the antitumor effects of TET for 21 days. Sora and TET were given by intraperitoneal injection or oral gavage.

Results: For SMMC7721 (IC_{50} = 22.5 μM) and PLC8024 (IC_{50} = 18.4 μM), TET (10, 20 μM) reduced colony number (0.68 ± 0.04- and 0.50 ± 0.04-fold, 0.56 ± 0.04- and 0.42 ± 0.02-fold), induced cell cycle arrest at G0/G1 stage (1.22 ± 0.03- and 1.39 ± 0.07-fold, 1.37 ± 0.06- and 1.55 ± 0.05-fold), promoted apoptosis (2.49 ± 0.26- and 3.63 ± 0.33-fold, 2.74 ± 0.42- and 3.73 ± 0.61-fold), and inactivated PI3K/AKT/mTOR signalling. Sora (10 μM) decreased cell proliferation, enhanced apoptosis, and inhibited PI3K/AKT/mTOR signalling, and these effects were further aggravated in the combination group. Activating PI3K/AKT/mTOR reversed the effects of TET on cell proliferation and Sora sensitivity. In the combination group, tumour volumes and weights were decreased to 202.3 ± 17.4 mm^3 and 151.5 ± 25.8 mg compared with Sora (510.6 ± 48.2 mm^3 and 396.7 ± 33.5 mg).

Discussion and conclusions: TET enhances Sora sensitivity by inactivating PI3K/AKT/mTOR, suggesting the potential of TET as a chemosensitizer in HCC.

ARTICLE HISTORY
Received 4 June 2021
Revised 27 October 2021
Accepted 7 December 2021

KEYWORDS
Liver cancer; systematic pharmacology; traditional Chinese medicine; PI3K/AKT signalling; cell cycle; apoptosis; chemosensitivity

Introduction

Hepatocellular carcinoma (HCC), a major histologic type of liver cancer, is the 7th most frequently occurring cancer and the 2nd leading cause of cancer-related deaths worldwide (Sung et al. 2021). As reported, HCC is predominant in Asian countries, especially in the East and South-East (Petrick et al. 2020). In addition to chronic infections with HBV and HCV, metabolic syndrome, obesity, type II diabetes mellitus, non-alcoholic fatty liver disease, alcohol consumption, and cigarette smoking are becoming the major contributors of HCC (McGlynn et al. 2021). Due to the inconspicuous symptoms in the early stage of this disease, most HCC patients are diagnosed at the advanced stage and miss the opportunity to receive surgical resection. Although significant advances have been made in HCC diagnosis and treatment, the 5-year relative survival rate for HCC patients is only 18% (Siegel et al. 2020). Developing novel palliative therapies are imperative to prolong life for advanced HCC patients.

Traditional Chinese Medicine (TCM) has long been selected as a complementary and alternative therapy for cancer patients following surgery, chemotherapy, and radiotherapy worldwide (Xiang et al. 2019). Natural compounds derived from herbs are considered ideal candidates for suppressing malignancies due to the advantages of multitarget and low toxicity (Dutta et al. 2019). Chinese herbal compounds have been elucidated as effective tumour suppressors in HCC *via* regulating cell proliferation, cell cycle, apoptosis, autophagy, cell senescence, epithelial-mesenchymal transition, metastasis, angiogenesis, immune function, and chemotherapy resistance (Hu et al. 2016). Tetrandrine (TET), a bisbenzylisoquinoline alkaloid isolated from the roots of *Stephania tetrandra* S. Moore (Menispermaceae), possesses different pharmacological activities, including anti-inflammatory, anti-allergic, antioxidative, antidiabetic, antimicrobial, and cardiovascular protective properties (Bhagya and Chandrashekar 2016). TET has been shown to exert antineoplastic effects in various human cancers by suppressing proliferation, migration, invasion, and angiogenesis; increasing apoptosis and autophagy; reversing multidrug resistance (MDR); and inducing radiation sensitization (Liu et al. 2016). For example, TET inhibits tumour growth in triple-negative breast cancer by inducing apoptosis (Wang CH et al. 2020). TET represses the migration and invasion of nasopharyngeal carcinoma cells *in vitro* by inactivating MAPK and RhoA signalling pathways (Wu et al. 2020). TET hinders proliferation and enhances apoptosis in neuroblastoma *in vitro* and *in vivo via* suppressing Hippo/YAP signalling (Zhao et al. 2019). TET decreases cell proliferation and promotes apoptosis in colon cancer by modulating BMP9/PTEN/PI3K/AKT

CONTACT Guoyong Chen chengy8530@163.com Department of Hepatobiliary and Pancreas Surgery, Henan Provincial People's Hospital, No. 7 Weiwu Road, Zhengzhou 450003, China

signalling (Zhou et al. 2021). It is also reported that the maximum non-cytotoxic dose of TET increases the sensitization of nasopharyngeal carcinoma cells to irradiation by inactivating MEK/ERK pathway and inducing autophagy (Wang J et al. 2020). To date, there have been several studies demonstrating the influence of TET on cell proliferation, apoptosis, and metastasis in HCC (Liu et al. 2011; Yu and Ho 2013; Zhang et al. 2018). Nevertheless, the therapeutic targets and signalling mechanisms underlying TET-mediated inhibition of HCC progression deserve to be systematically investigated.

Sorafenib (Sora), a multikinase inhibitor, is the only systemic agent approved for HCC patients at an advanced stage (Keating 2017). During long-term chemotherapy, acquired drug resistance severely limits the clinical response and impairs the survival rate of patients. TCM-derived natural compounds are demonstrated to reverse MDR of orthodox chemotherapeutic drugs by affecting drug efflux, metabolism enzymes, DNA damage repair, and anti-apoptosis pathways (Lou et al. 2018). Thus, it is of great value to unearth novel natural compounds as effective chemosensitizers to improve the efficacy of Sora in HCC treatment.

Network pharmacology has been widely applied to explore the basic pharmacological effects and molecular mechanisms of TCM or natural compounds in diseases by constructing drug-disease-target networks (Poornima et al. 2016; Zhang et al. 2019). In the current study, we used a network pharmacology approach to uncover the potential targets and pathways of TET against HCC. Additionally, *in vitro* and *in vivo* experiments were performed to validate the biological functions and mechanisms of TET as an anticancer agent and Sora sensitizer in HCC.

Materials and methods

Collecting the potential targets of TET against HCC

The potential targets of TET were retrieved from TargetNet (https://targetnet.scbdd.com), SwissTargetPrediction (https://www.swisstargetprediction.ch/), TCMSP (https://old.tcmsp-e.com/tcmsp.php), and PharmMpper (http://www.lilab-ecust.cn/pharmmapper/submitfile.html). HCC-related targets were collected by searching three databases including TTD (http://db.idrblab.net/ttd/), GeneCards (https://www.genecards.org/), and OncoDB.HCC (http://oncodb.hcc.ibms.sinica.edu.tw/index.htm).

Network construction

The component-target-disease (CTD) interaction was established by inputting 47 common targets of TET and HCC into the Cytoscape software. The protein-protein interaction (PPI) network for the overlap genes between TET targets and HCC targets was constructed with the online String database and analyzed by the Cytoscape software.

GO and KEGG pathway enrichment

GO function annotation and KEGG pathway enrichment were performed using the OmicShare cloud platform (www.omicshare.com/). The enriched terms were considered statistically significant when $p < 0.05$. The top 20 biological processes (BP), molecular functions (MF), cellular components (CC), and KEGG pathways were output in the form of bubble charts using bioinformatics tool (http://www.bioinformatics.com.cn/).

Cell culture

Human HCC cell lines (SMMC7721 and PLC8024), normal human hepatic cells L02, and human mammary epithelial cell HBL-100 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were maintained in a DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated FBS and 100 U/ml penicillin-streptomycin. Cells were routinely cultured at 37 °C in an incubator with 5% CO_2.

Cell treatment

To observe the effects of TET or Sora on HCC, SMMC7721 and PLC8024 cells were treated with indicated doses of TET (0, 2.5, 5, 10, 20, 40, and 80 μM) (Liu et al. 2011) or Sora (0, 2.5, 5, 10, 20, 30, and 40 μM) (Gan et al. 2021). To activate PI3K/AKT signalling, a PI3K agonist 740Y-P (MedChem Express, Monmouth Junction, NJ, USA) was used at a final concentration of 10 μM (Gong et al. 2019) to pretreat SMMC7721 and PLC8024 cells for 3 h.

CCK-8 assay

Cell viability was assessed by using Cell Counting Kit-8 (CCK-8; MedChemExpress, Monmouth Junction, NJ, USA). SMMC7721 and PLC8024 cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. Then, TET or Sora was added into each well at the indicated concentration for 24 or 48 h of incubation. After the addition of 10 μL of CCK-8 solution, cells were incubated for another 3 h at 37 °C. Optical density (OD) was read at 450 nm with a microplate reader (Labsystems, Helsinki, Finland). The half-maximal inhibitory concentration (IC_{50}) of TET or Sora was calculated by plotting dose-response curves.

Colony formation assay

SMMC7721 and PLC8024 cells were inoculated into 6-well plates at a density of 5×10^2 cells/well. After overnight attachment, cells were exposed to TET or Sora for 48 h at indicated doses. Then, cells were cultured in a fresh medium for 12 days to form colonies. Thereafter, cells were fixed with 20% ethanol and stained with 0.5% crystal violet. The number of colonies containing more than 50 cells was manually counted.

Cell cycle assay

SMMC7721 and PLC8024 cells were seeded into 6-well plates and treated with specified doses of TET or Sora for 48 h. Then, cells were trypsinized, washed with PBS, and fixed in prechilled 70% ethanol at 4 °C overnight. Next, cells were stained with propidium iodide (PI) containing RNase A at room temperature for 30 min in the dark. For each measure, 10,000 events were analyzed. The samples were subjected to FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, USA) to analyze cell cycle distribution.

Flow cytometry assay of apoptosis

SMMC7721 and PLC8024 cells were seeded into 6-well plates and exposed to indicated doses of TET or Sora for 48 h. Cells

were digested with trypsin, washed with PBS, and resuspended in binding buffer. Thereafter, cells were stained with 5 µL Annexin V-FITC and 5 µL PI at room temperature for 15 min, followed by apoptosis analysis with a FACSCalibur flow cytometer (BD Biosciences). For each sample, at least 10,000 events were recorded and analyzed.

Western blot assay

Total protein was extracted from HCC cells using RIPA lysis buffer and protein concentration was determined by a BCA Protein Assay Kit (Beyotime, Shanghai, China). The extracted proteins (40 µg) were separated on 10% SDS-PAGE gels, transferred onto PVDF membranes, and blocked in 5% non-fat milk for 1 h at room temperature. Then, the membranes were incubated with the primary antibodies against PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, PCNA, Cyclin D1, Bax, Bcl-2, or GAPDH overnight at 4 °C. After washing, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Finally, ECL-Plus reagent (Millipore, Billerica, MA, USA) was used to visualize the immunoblots. GAPDH was used as a loading control to normalize the expression of different proteins. Primary antibodies (PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, and GAPDH) were purchased from Abcam (Cambridge, MA, USA). Primary antibodies (PCNA, Cyclin D1, Bax, and Bcl-2) were obtained from Cell Signalling Technology (Danvers, MA, USA).

In vivo *xenograft assays*

Five-week-old male BALB/c nude mice were purchased from Shanghai SLAC Laboratory Animal Centre (Shanghai, China). SMMC7721 cells (5×10^6) in 200 µL PBS were subcutaneously injected into the right armpit of each mouse. On day 5, mice were randomly divided into four groups ($n = 4$ in each group): Control group (PBS), Sora group (10 mg/kg Sora) (Gan et al. 2021), TET group (50 mg/kg TET) (Liu et al. 2011) and TET + Sora group (10 mg/kg Sora plus 50 mg/kg TET). Sora was given by intraperitoneal injection and TET was administrated by oral gavage every other day. During the experiment period, tumour sizes were measured every 4 days and tumour volumes were calculated following the formula: $V = (\text{Length} \times \text{Width}^2)/2$. In the end, mice were killed and tumours were removed for weight measurement. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Henan Provincial People's Hospital and complied with the National Institutes of Health guide for the care and use of laboratory animals.

Immunohistochemistry (IHC) assay

IHC assay was conducted according to the procedures depicted in a previous document (Yang J et al. 2019). In brief, 5 µm thick, formalin-fixed, and paraffin-embedded sections of tissue specimen were prepared. Immunohistochemical staining for proliferation marker PCNA was performed by using an anti-PCNA antibody (Abcam, ab92552).

Statistical analysis

Data from three independent experiments were shown as mean ± standard deviation. Each experiment was performed in triplicate. One-way analysis of variance (ANOVA) was used for comparison among multiple groups. The difference was considered to be statistically significant when $p < 0.05$.

Results

Identifying targets of TET against HCC

By mining databases including TargetNet, SwissTargetPrediction, TCMSP, and PharmMpper, a total of 229 targets of TET were collected after deleting the duplicates. By retrieving the data from TTD, GeneCards, and OncoDB.HCC, 717 HCC-related targets were collected after eliminating the repetitive genes. According to the intersection presented by the Venn diagram, 47 common targets were found to be associated with TET and HCC (Figure 1(A)).

Network construction

To elucidate the interaction between TET, HCC, and targets, a 'CTD' interaction network was generated *via* inputting TET, targets, and HCC into the Cytoscape software (Figure 1(B)). This network consisted of 48 nodes (one disease, one compound, and 46 targets). The result suggested that TET might exert regulatory effects in HCC by targeting multiple genes. To clarify the interaction between these 47 shared targets, a PPI network was constructed with a String database and analyzed by Cytoscape software. As presented in Figure 1(C), the PPI network contained 46 nodes and 350 edges. Through network topology, the second screening ended up with 20 nodes and 164 edges. Finally, 20 hub genes with a node degree greater than the average value of 15.2 were identified. The hub genes with a higher degree might be important for the pharmacological processes. The therapeutic effects of TET in HCC might be largely attributed to the top five targets, including CCND1, TP53, EGFR, MTOR, and SRC.

GO function and KEGG pathway enrichment

GO function annotation was performed to investigate the crucial biological functions of 20 core targets. The core genes were enriched in 1948 biological processes (BP), and there were 184 BP with gene count >10. As shown in Figure 2(A), the top 15 GO items related to BP were mainly cellular response to chemical stimulus, regulation of cell death, response to oxygen-containing compound, negative regulation of the apoptotic process, and positive regulation of the cellular metabolic process. The core genes were enriched in 199 processes related to molecular functions (MF), mainly including protein kinase binding, enzyme binding, protein kinase activity, transcription factor binding, and phosphotransferase activity (Figure 2(B)). The core genes were enriched with 114 cellular components (CC), mainly related to protein-containing complex, transferase complex, cyclin-dependent protein kinase holoenzyme complex, nucleoplasm, and cytosol (Figure 2(C)). To explore the potential therapeutic mechanisms of TET in HCC, we performed a KEGG pathway enrichment analysis of these 20 core targets. The most significantly enriched 15 pathways of TET involved in HCC were presented in Figure 2(D). The pathways in cancers showed the largest number of involved targets (14 counts). PI3K-AKT pathway is one of the most frequently dysregulated pathways in cancer and is responsible for multiple biological processes, such as metabolism, metastasis, proliferation, apoptosis, and survival

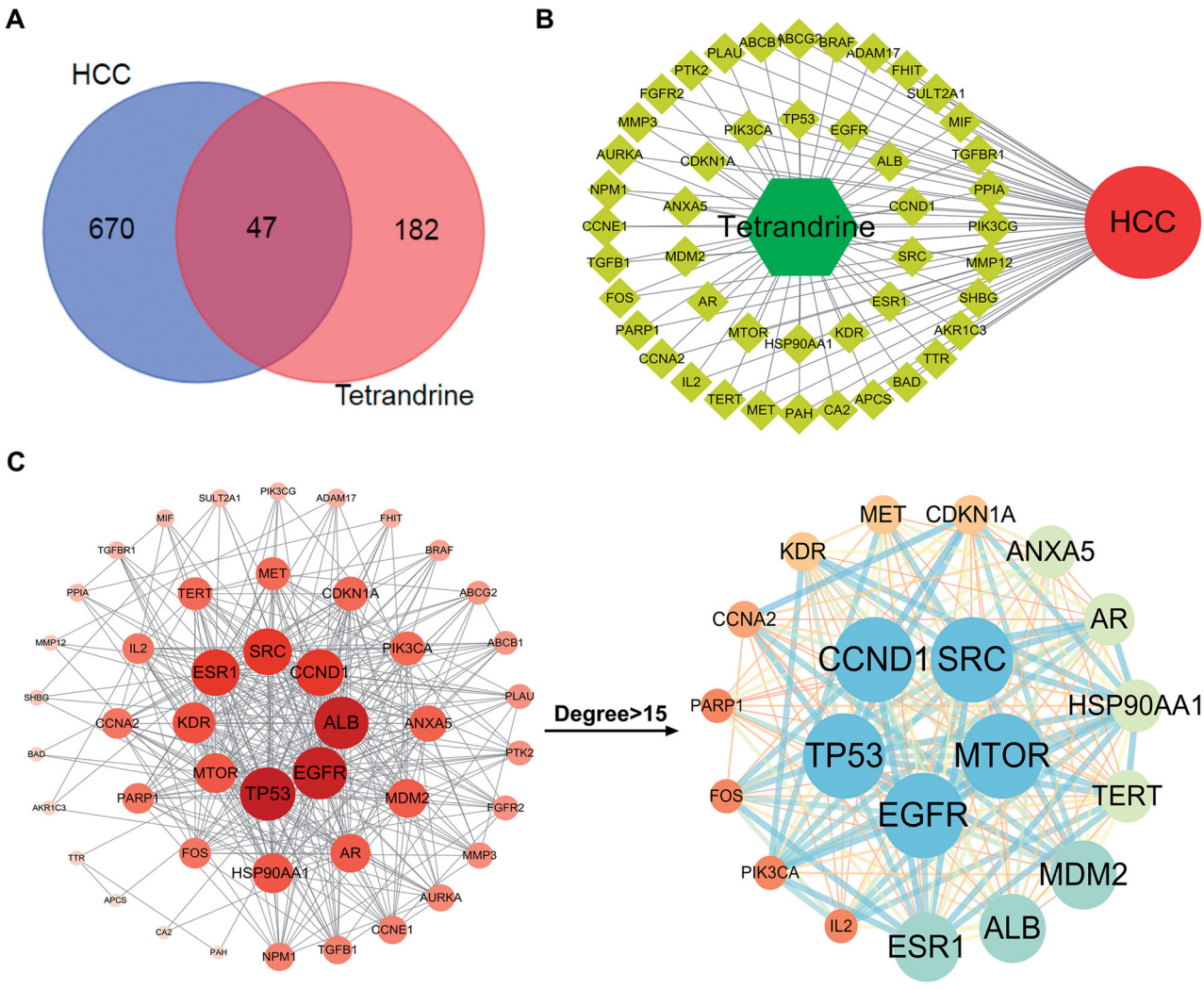

Figure 1. Network pharmacology construction of TET against HCC. (A) Venn diagram was used to exhibit the overlap of HCC- and TET-related targets. (B) Network of TET targets and HCC targets. The hexagon is a represent of compound TET. The square is a represent of 46 common targets. The circle is a represent of HCC. (C) PPI network and topological screening.

associated with tumorigenesis (Janku et al. 2018). Based on the above results, we speculated that TET might increase drug sensitivity in HCC by inducing apoptosis *via* PI3K-AKT signalling.

TET inhibits cell proliferation and induces apoptosis in HCC

For evaluating the effects of TET on HCC as predicted from network pharmacology analysis, SMMC7721 and PLC8024 cells were exposed to different concentrations of TET (0, 2.5, 5, 10, 20, 40, and 80 μM) for 24 and 48 h. CCK-8 assay showed that TET significantly repressed cell viability in a dose- and time-dependent manner (Figure 3(A)). The IC_{50} values of TET for SMMC7721 and PLC8024 cells were 31.2 and 25 μM at 24 h, and 22.5 and 18.4 μM at 48 h, respectively. After treatment with TET (10 or 20 μM) for 48 h, no cytotoxic effects were observed in normal human hepatic cells (L02) and human mammary epithelial cells (HBL100) (Figure 3(B)). Thus, cell cycle, colony formation, and apoptosis assays were conducted in HCC cells after treatment with 10 or 20 μM TET for 48 h. As presented in Figure 3(C), colony-forming ability of HCC cells was significantly decreased in the presence of TET. The colony number was 161 ± 10, 110 ± 10, 80 ± 9 for SMMC7721 cells and 170 ± 9, 95 ± 7, 72 ± 6 for PLC8024 cells at 0, 10, and 20 μM TET, respectively. Moreover, treatment of HCC cells with TET led to a significant cell cycle arrest at G0/G1 stage (Figure 3(D)). The percentage of the cells in G0/G1 phase was 54.8 ± 2.4, 66.8 ± 1.5, 76.2 ± 0.8% for SMMC7721 cells and 48.8 ± 1.5, 67.0 ± 1.5, 76.9 ± 0.9% for PLC8024 cells at 0, 10, and 20 μM TET, respectively. Subsequently, the effects of TET on HCC cell apoptosis were detected by flow cytometry assays. It turned out that the cell apoptotic rate was significantly increased by TET treatment (Figure 3(E)). The apoptotic rate was 5.4 ± 0.6, 13.4 ± 1.3, 19.5 ± 0.9% for SMMC7721 cells and 5.9 ± 0.5, 16.1 ± 1.2, 21.9 ± 1.7% for PLC8024 cells at 0, 10, and 20 μM TET, respectively. As demonstrated by western blot, TET treatment decreased the expression of PCNA, Cyclin D1, and Bcl-2, but increased the expression of Bax in both SMMC7721 and PLC8024 cells (Figure 3(F)). Taken together, TET suppressed cell proliferation and enhanced apoptosis in HCC *in vitro*.

TET inactivates PI3K/AKT/mTOR signalling in HCC cells

The effects of TET on PI3K/AKT/mTOR signalling were examined in HCC cells. Western blot assays were performed to measure the expression levels of proteins associated with PI3K/AKT/mTOR signalling in HCC cells after treatment with TET (0, 10,

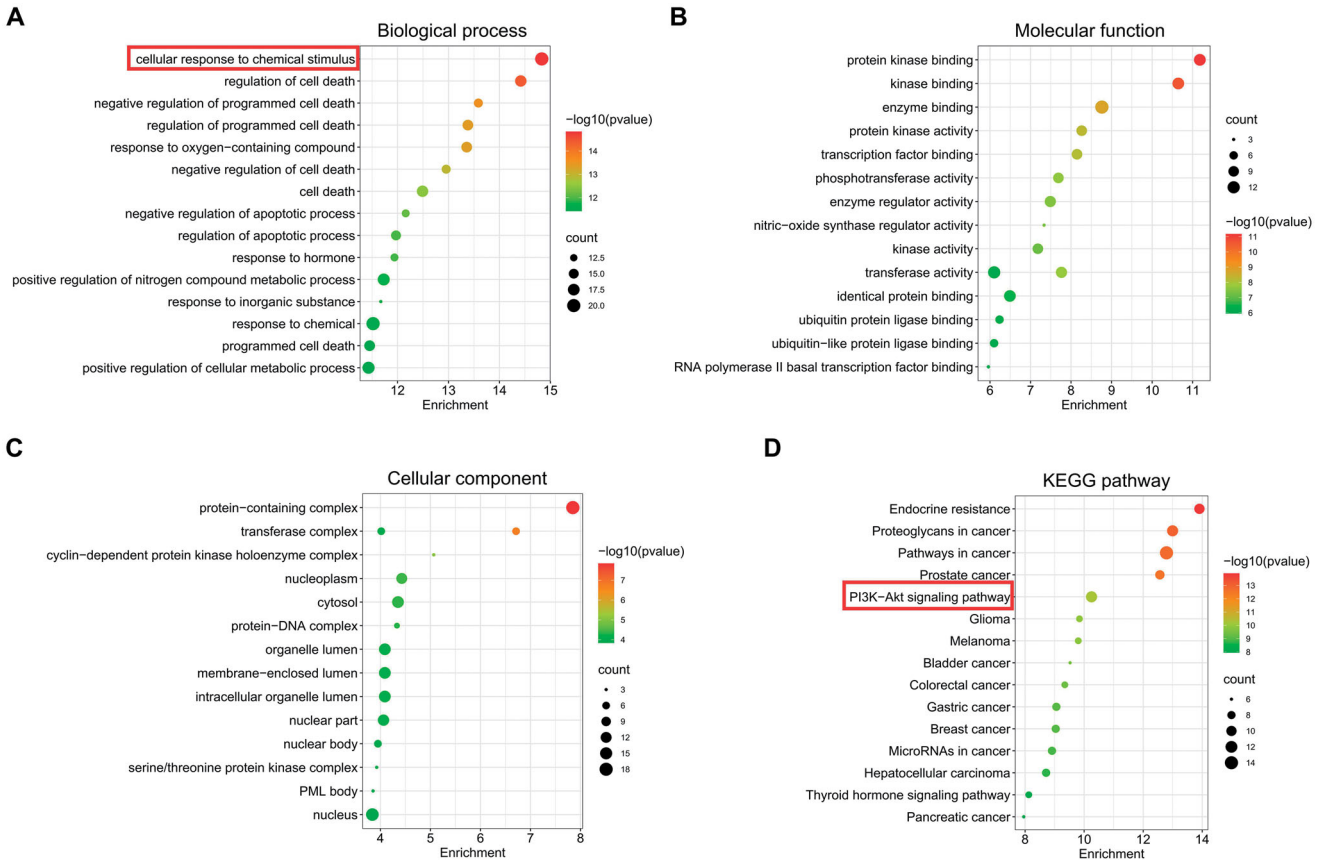

Figure 2. GO and KEGG pathway enrichment analysis of 20 core targets. (A–C) GO enrichment analysis of biological processes (A), molecular functions (B), and cellular components (C) of core genes for TET against HCC. (D) KEGG pathway enrichment analysis of core genes for TET against HCC.

20) for 48 h. TET treatment inhibited the phosphorylation level of PI3K, AKT, and mTOR, while total PI3K, AKT, and mTOR levels were not significantly changed (Figure 4(A,B)). These data indicated that TET repressed PI3K/AKT/mTOR signalling pathway in HCC cells.

TET suppresses cell proliferation and promotes apoptosis in HCC by inactivating PI3K/AKT/mTOR pathway

To determine whether PI3K/AKT/mTOR pathway was involved in TET-induced proliferation inhibition and apoptosis, HCC cells were pre-treated with 740Y-P (10 μM) for 3 h before treatment with TET (20 μM) for 48 h. TET-induced decrease of p-PI3K, p-AKT, and p-mTOR protein expression was significantly reversed in the presence of 740Y-P (Figure 5(A,B)). The colony number in the TET group was reduced in SMMC7721 (95 ± 13 *vs.* 174 ± 10, $p < 0.001$) and PLC8024 (76 ± 11 *vs.* 156 ± 11, $p < 0.001$) cells, and then increased after being treated with 740Y-P in SMMC7721 (147 ± 14 *vs.* 95 ± 13, $p < 0.01$) and PLC8024 (129 ± 11 *vs.* 76 ± 11, $p < 0.01$) cells (Figure 5(C)). Similarly, TET-mediated cell cycle arrest was obviously weakened due to the addition of 740Y-P (Figure 5(D)). Compared with the single TET group, TET combined with 740Y-P resulted in a significant reduction of apoptosis (12.9 ± 1.47 *vs.* $24.77 \pm 1.92\%$ in SMMC7721, 13.63 ± 2.35 *vs.* $28.27 \pm 3.17\%$ in PLC8024) (Figure 5(E,F)). Consistently, TET-induced decrease of PCNA, Cyclin D1 and Bcl-2, and increase of Bax were abated in response to 740Y-P (Figure 5(G,H)). The above data confirmed that TET inhibited HCC progression by inactivating the PI3K/Akt/mTOR pathway.

TET sensitizes HCC cells to sora in vitro

Then, we further explored the effect of TET on Sora sensitivity in HCC. SMMC7721 and PLC8024 cells were treated with indicated doses of Sora for 48 h. As shown in Figure 6(A), Sora treatment led to an inhibition of cell viability. The IC_{50} values of Sora for SMMC7721 and PLC8024 cells were 20.2 and 15.7 μM at 48 h, respectively. According to the IC_{50} values of TET and Sora, HCC cells were concurrently treated with 10 μM TET and 10 μM Sora for 48 h. Compared with the Sora group, the colony number of HCC cells in the combination group (Sora + TET) was significantly decreased (73 ± 6 *vs.* 143 ± 10 in SMMC7721, 65 ± 11 *vs.* 130 ± 11 in PLC8024) (Figure 6(B)). Similarly, the cell percentage at the G0/G1 stage was increased in the combination group compared to the single Sora treatment group (78.1 ± 1.9 *vs.* $62.0 \pm 1.7\%$ in SMMC7721, 75.5 ± 1.5 *vs.* $60.8 \pm 1.6\%$ in PLC8024) (Figure 6(C)). Also, Sora-induced apoptosis was further enhanced after concomitant treatment with TET and Sora (34.90 ± 0.54 *vs.* $17.70 \pm 0.56\%$ in SMMC7721, 39.87 ± 3.10 *vs.* $18.43 \pm 1.99\%$ in PLC8024) (Figure 6(D)). Overall, TET could induce the chemosensitivity of HCC cells to Sora.

TET increases the sensitivity of HCC cells to sora through inactivating PI3K/AKT/mTOR pathway

Next, we tried to clarify whether PI3K/AKT/mTOR signalling was involved in TET-induced chemosensitivity. Compared with the Sora group, the combination of Sora and TET dramatically lowered the phosphorylation level of PI3K, AKT, and mTOR (Figure 7(A)). Moreover, PI3K activator 740Y-P restored the cell viability inhibited by the combination of Sora and TET (Figure

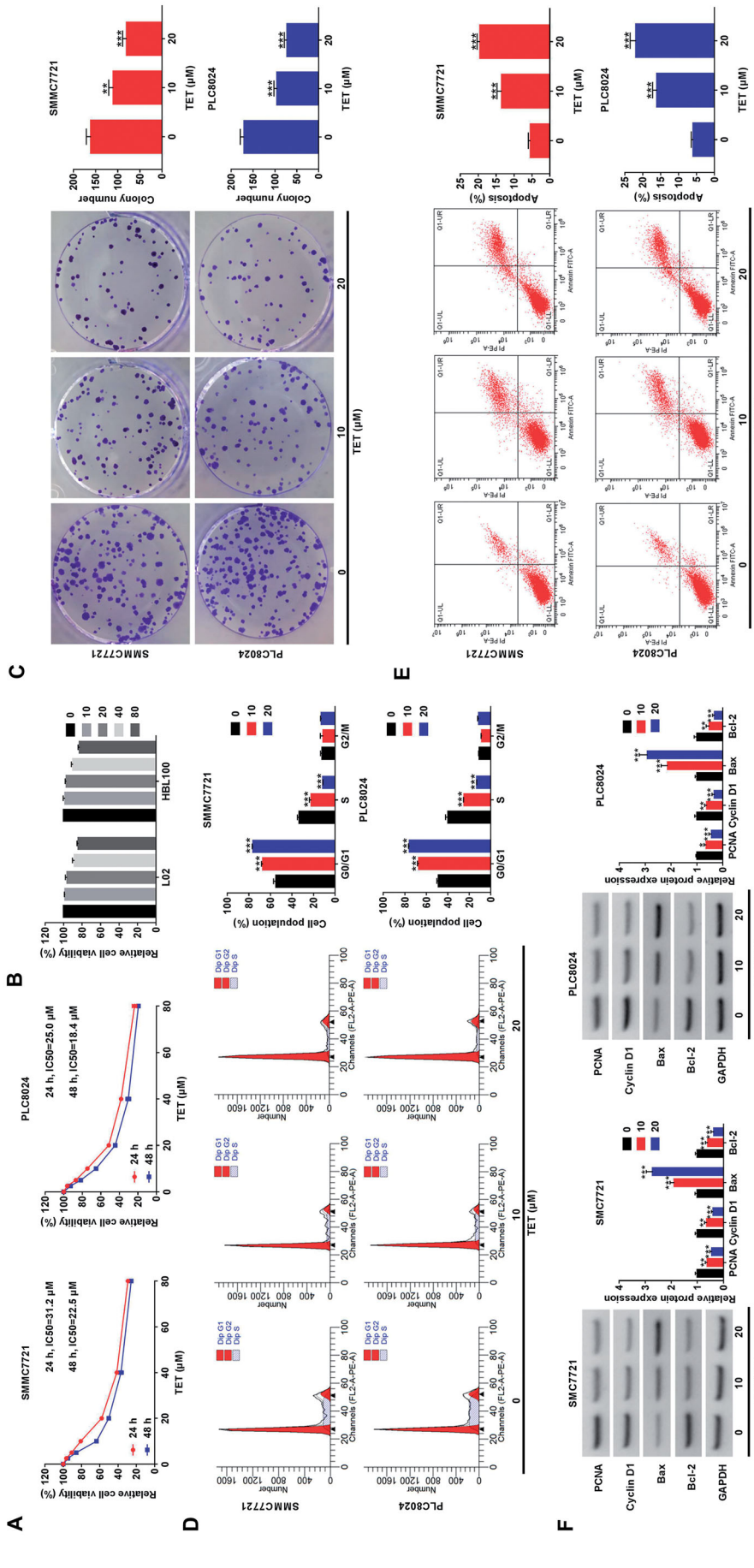

Figure 3. TET suppresses cell proliferation and induces apoptosis in HCC. (A) CCK-8 assay was used to measure the viability of SMMC7721 and PLC8024 after treatment with TET (0–80 μM) for 24 and 48 h. (B) Effects of TET on L02 and HBL100 were determined by CCK-8 assay. (C-F) SMMC7721 and PLC8024 were treated with 10 or 20 μM TET for 48 h. (C) Colony formation assays. (D) Cell cycle distribution was examined by flow cytometry. (E) Flow cytometry assay of apoptosis. (F) Western blot assays were applied to detect the protein expression of PCNA, Cyclin D1, Bcl-2, and Bax. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* 0 μM group.

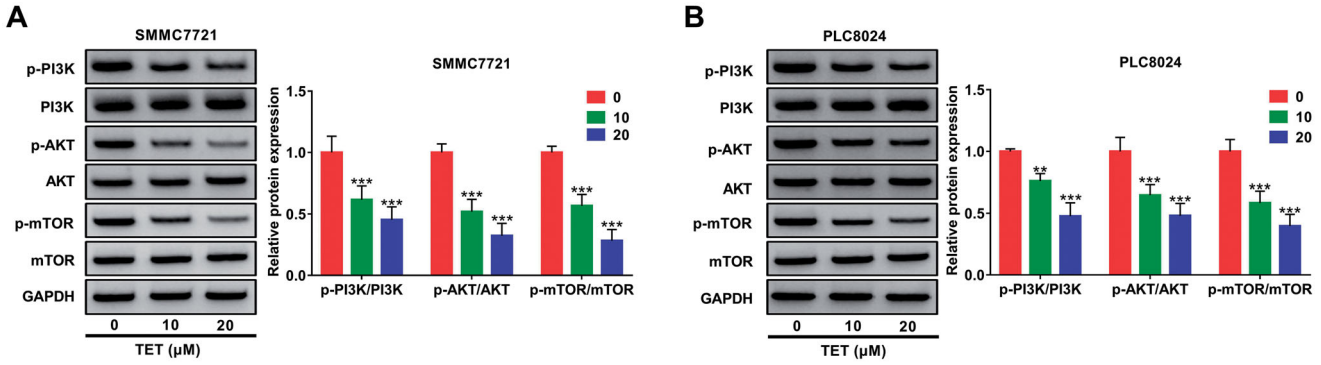

Figure 4. TET inactivates PI3K/AKT/mTOR signalling pathway in HCC cells. (A,B) The protein level of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR were determined in HCC cells after treatment with 10 or 20 μM TET for 48 h. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* 0 μM group.

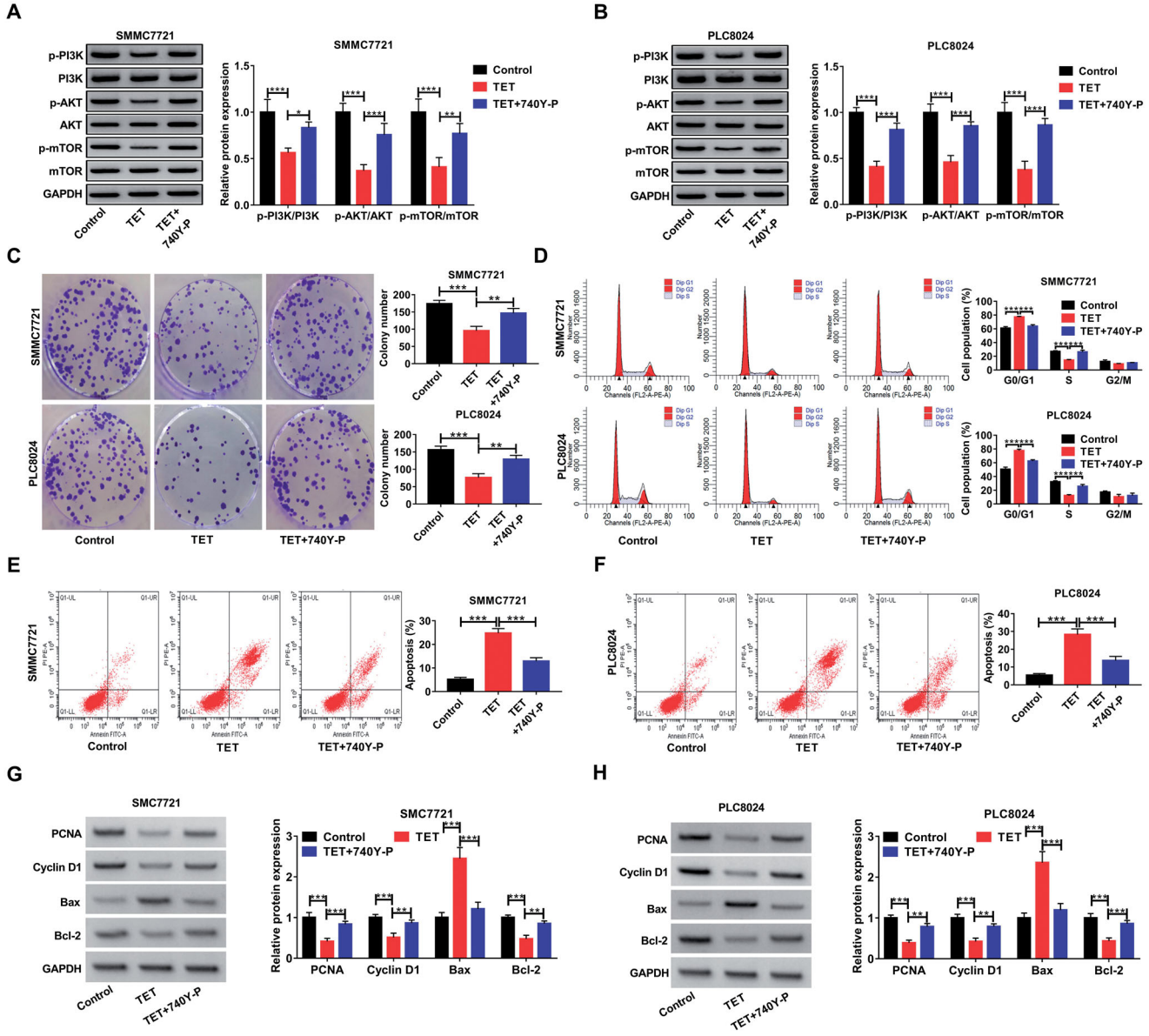

Figure 5. TET represses PI3K/AKT/mTOR signalling pathway to hinder cell proliferation and facilitate apoptosis in HCC. (A–H) HCC cells were pre-treated with 740Y-P (10 μM) for 3 h before treatment with TET (20 μM) for 48 h, followed by (A,B) western blot analysis of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR protein expression; (C) detection of colony-forming ability; (D) flow cytometry analysis of cell cycle distribution; (E,F) flow cytometry analysis of apoptosis; (G,H) western blot analysis of PCNA, Cyclin D1, Bcl-2, and Bax protein levels. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control or TET group.

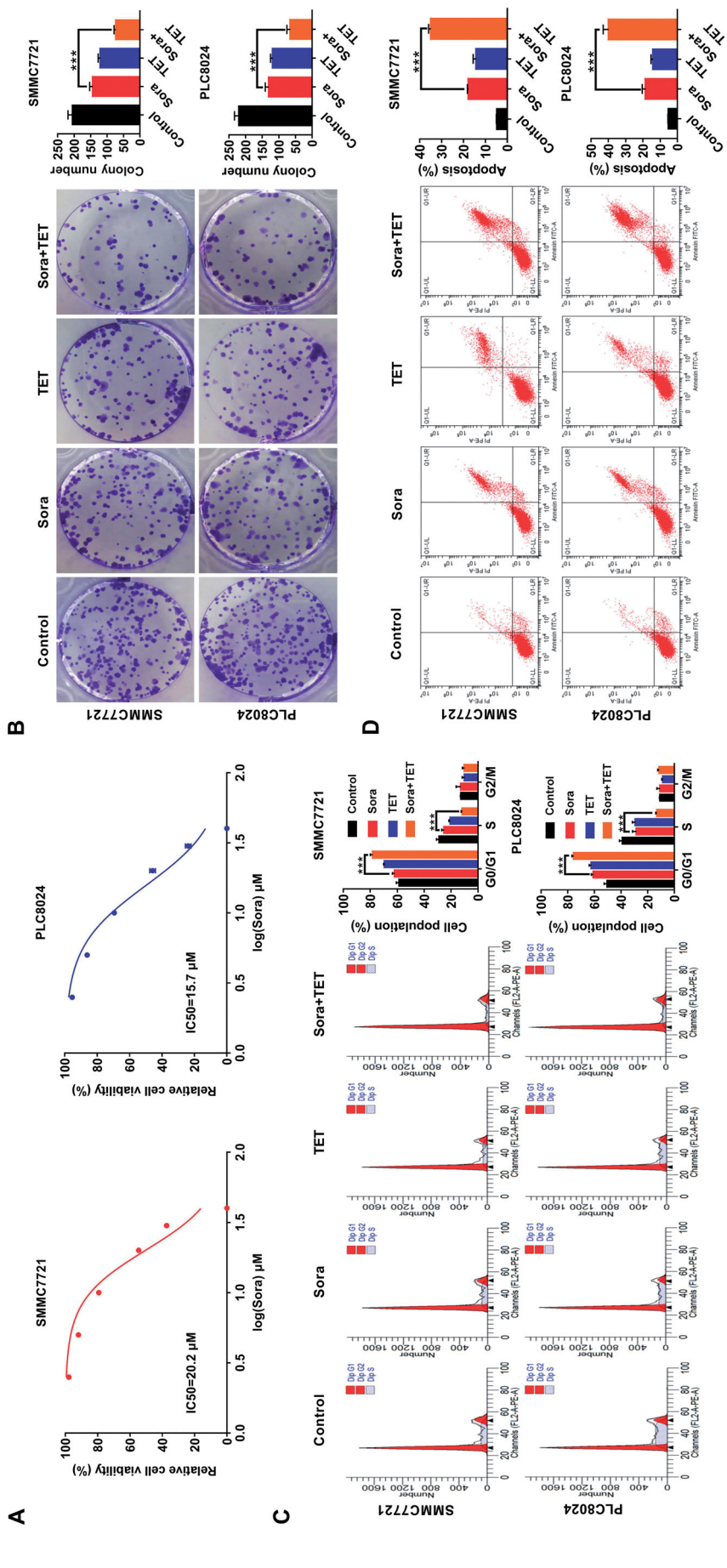

Figure 6. TET increases Sora sensitivity in HCC cells. (A) CCK-8 assay was used to detect HCC cell viability after incubation with Sora (0–40 μM) for 48 h. (B–D) HCC cells were treated with 10 μM Sora, 10 μM TET or (10 μM Sora + 10 μM TET) for 48 h, followed by measurement of colony-forming potential (B), cell cycle distribution (C), and apoptotic rate (D). $^{***}p < 0.001$ *vs.* Sora group.

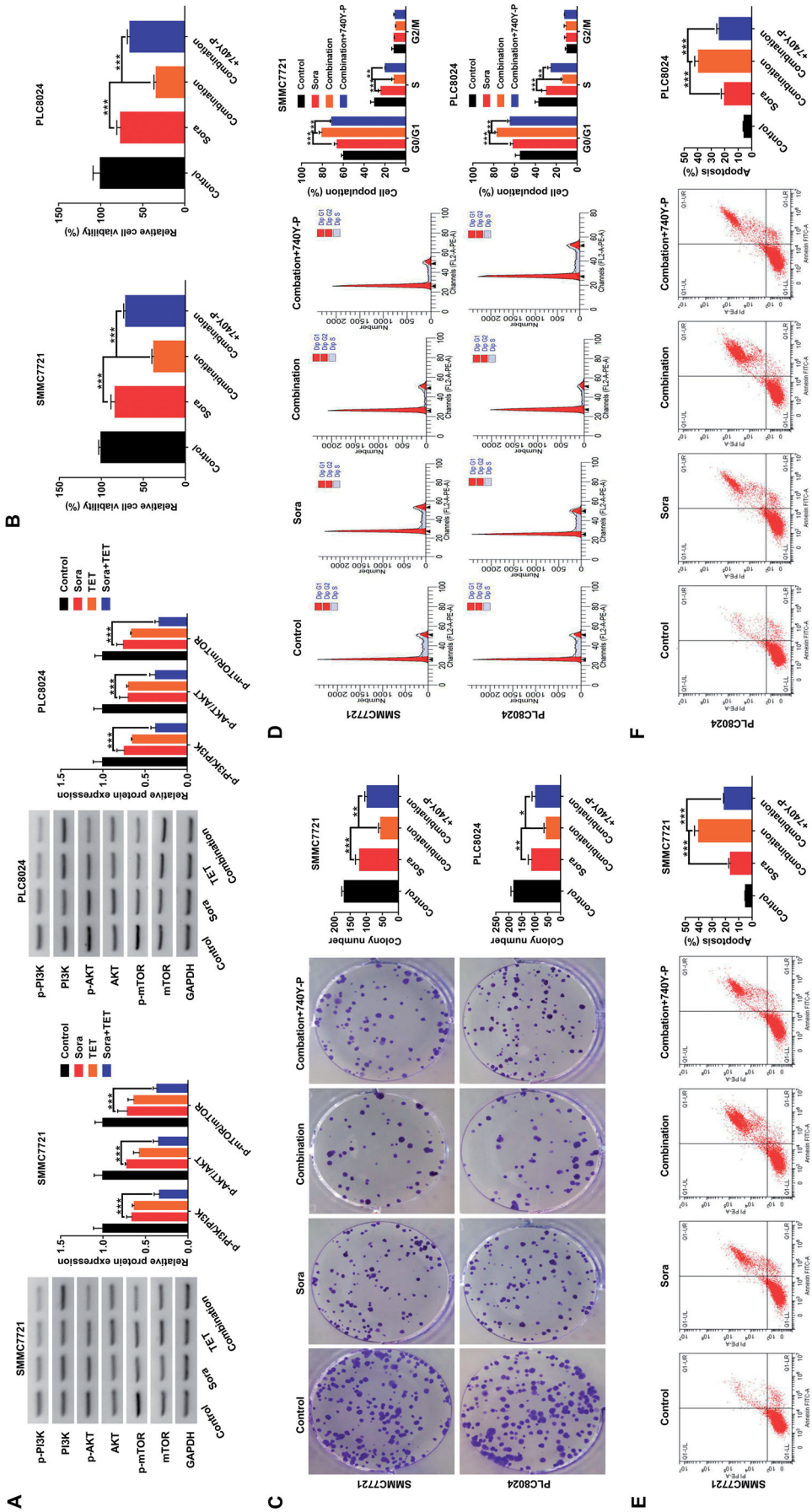

Figure 7. TET increases the sensitivity of HCC cells to Sora *via* inactivating PI3K/AKT/mTOR signalling. (A) Western blot assays were used to detect the expression level of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR in HCC cells from Control, Sora (10 μM Sora), TET (10 μM TET), and Combination (10 μM Sora + 10 μM TET) group. (B–D) HCC cells were treated with 10 μM Sora, or (10 μM Sora + 10 μM TET) with or without 10 μM 740Y-P, followed by determination of cell viability (B), colony-forming ability (C), cell cycle distribution (D), and apoptotic rate (E,F). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* Sora or combination group.

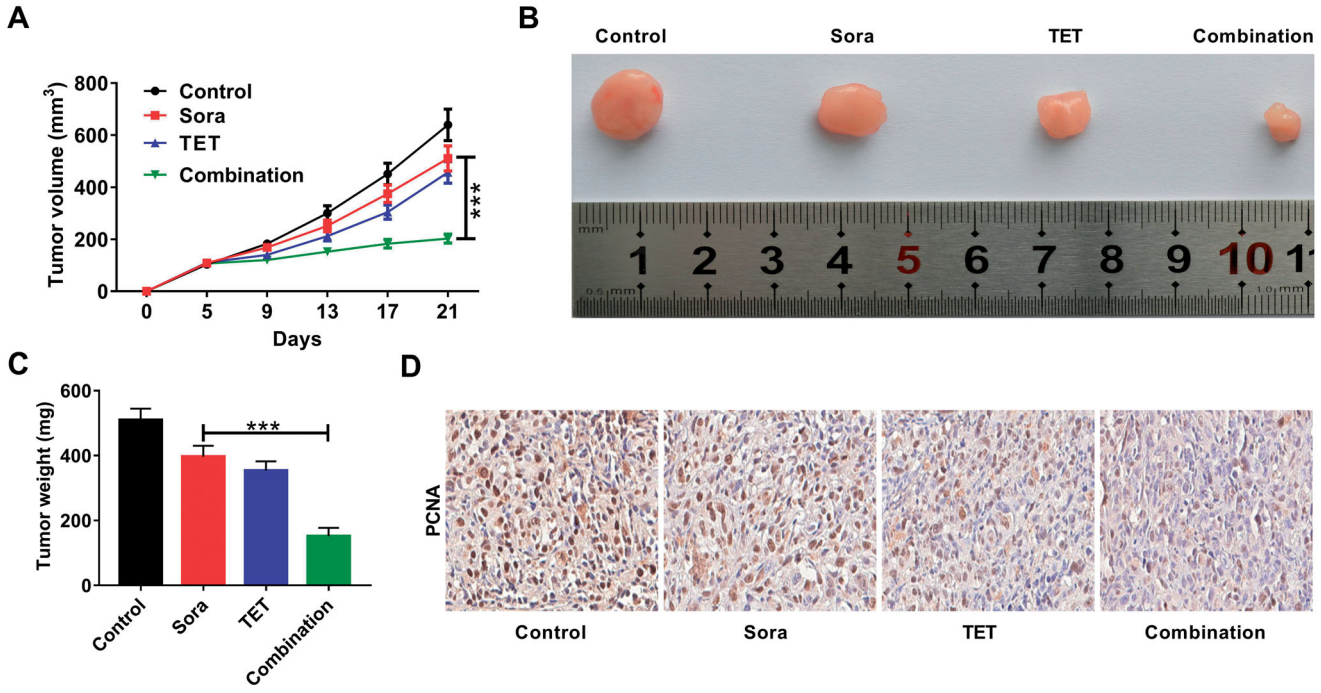

Figure 8. TET sensitizes HCC cells to Sora *in vivo*. SMMC7721 cells were inoculated into mice to construct a xenograft model. At day 5, mice were administered with PBS, Sora (10 mg/kg), TET (50 mg/kg) or TET + Sora (10 mg/kg Sora plus 50 mg/kg TET) every other day. (A) Tumour sizes were monitored at indicated time points. (B) Representative tumour images in different groups. (C) Tumour weights were determined at the end of the experiments. (D) IHC assays were performed to detect PCNA expression in tumour tissues. $^{***}p < 0.001$ *vs.* Sora group.

7(B)). In comparison with the combination group, the colony-forming ability was recovered following the addition of 740Y-P (Figure 7(C)). Consistently, cell cycle arrest induced by Sora and TET was significantly abated due to 740Y-P (Figure 7(D)). Additionally, cell apoptosis in the combination group was greatly reversed in the presence of 740Y-P (Figure 7(E,F)). In summary, TET sensitized HCC cells to Sora by inactivation of the PI3K/AKT/mTOR pathway.

TET sensitizes HCC cells to sora in vivo

Tumour xenograft in mice was established to confirm the effects of TET on HCC progression *in vivo*. Tumour volume in Sora or TET group was reduced to 79.9 and 71.5% of that in the control group, which was further decreased to 31.6% in the Sora plus TET group (Figure 8(A)). Compared with the control group, Sora or TET resulted in decreased tumour weight (396.7 ± 33.5 *vs.* 510.2 ± 34.5 mg, 353.8 ± 28.5 *vs.* 510.2 ± 34.5 mg). In the combination group, we observed the minimal tumour weight (151.5 ± 25.8 *vs.* 510.2 ± 34.5 mg) (Figure 8(B,C)). As demonstrated by IHC assays, Sora or TET injection resulted in a dramatic decline of PCNA expression, while this effect was further aggravated by a combination of Sora and TET (Figure 8(D)). These data suggested that TET repressed tumour growth and increased Sora sensitivity in HCC *in vivo*.

Discussion

HCC is a malignant tumour with high morbidity and mortality worldwide (Yang JD et al. 2019). Surgical resection and transplantation are considered the cornerstone of therapy for HCC patients at an early stage, while locoregional therapy and Sora are the main treatments for advanced disease (Grandhi et al. 2016). Nevertheless, the effects produced by these therapeutic methods are still unsatisfactory. Herbal medicines and natural plant products have been used as potentially therapeutic and chemopreventive agents for HCC for a long time due to their pleiotropic abilities to scavenge free radicals, repress cell growth, and promote apoptosis (Rawat et al. 2018). In this study, a system pharmacology approach was utilized to reveal the core targets, biological functions, and pathways of TET against HCC. Further experimental verification showed that TET suppressed tumour progression and enhanced Sora sensitivity by inactivating the PI3K/AKT/mTOR signalling in HCC both *in vitro* and *in vivo*.

TET was proved to possess definite antitumor activities in diverse human cancers by modulating apoptosis, autophagy, cell cycle arrest, cell proliferation, migration, and invasion (Luan et al. 2020). Here, 47 common targets associated with TET and HCC were acquired by virtue of public databases. Through network topological screening, 20 hub genes were found to be potentially involved in the anticancer role of TET in HCC. Based on the degree value, CCND1, TP53, EGFR, MTOR, and SRC were revealed as the top five key targets. GO function and KEGG enrichment assays discovered that TET might inhibit HCC progression by increasing drug sensitivity and inducing apoptosis *via* regulating PI3K/AKT signalling pathways.

To validate our hypothesis, a series of function experiments were carried out. The result displayed that TET inhibited cell proliferation, colony formation, and cell cycle progression, and promoted apoptosis in a dose-dependent manner. Consistently, TET treatment resulted in the decrease of PCNA, Cyclin D1, and Bcl-2 expression, while increased Bax expression. Also, TET injection significantly lowered tumour growth in mice. These data suggested the tumour-suppressive effect of TET on HCC. Similar to our data, Liu et al. (2011) elucidated that TET could induce HCC cells to undergo apoptosis by inducing reactive oxygen species (ROS) accumulation and suppressing AKT activity. Yu and Ho (2013) demonstrated that TET inhibited HCC cell

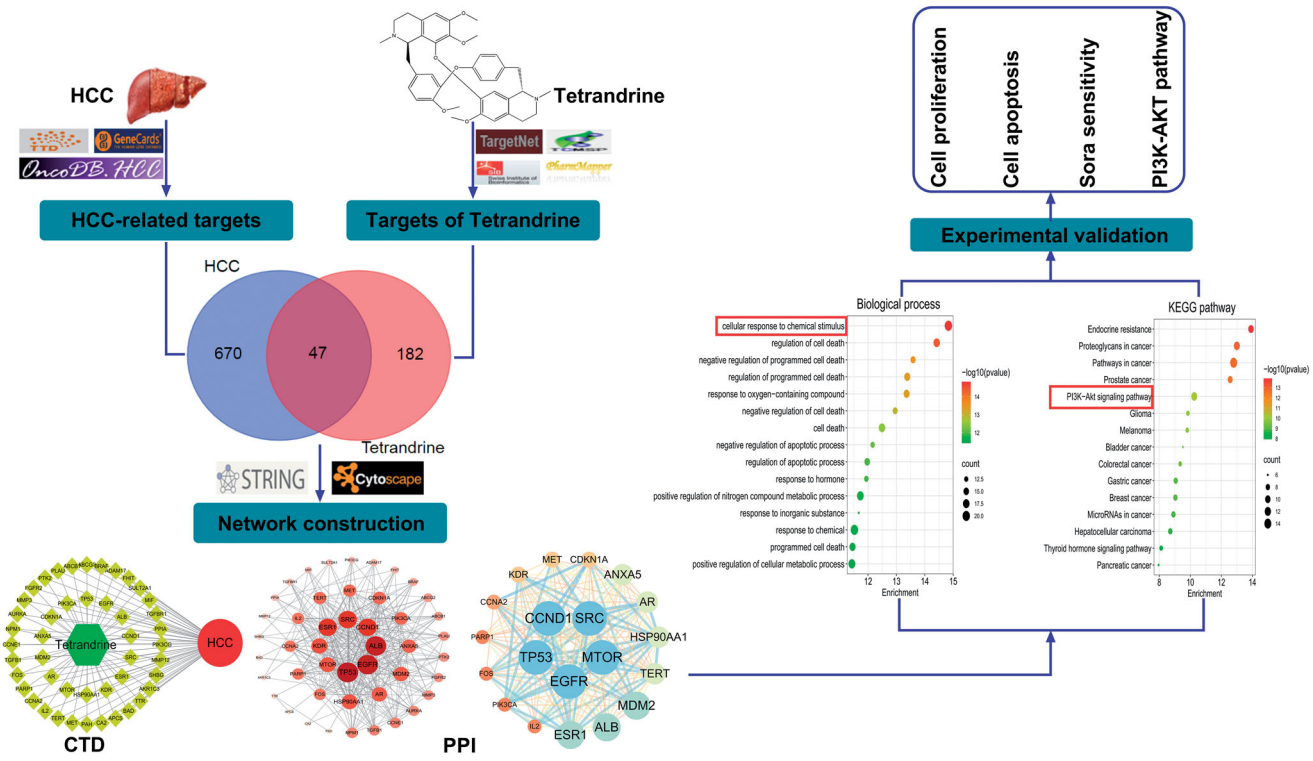

Figure 9. A flow diagram displaying the system pharmacology method for elucidating the action mechanism of TET in HCC *via* integrating target screening, network construction, and experimental validation.

growth *via* modulating the caspase pathway and G2/M phase. Zhao et al. (2018) discovered that TET increased the radiosensitization of HCC cell lines by inducing apoptosis and cell cycle arrest at least partly *via* down-regulating PA28γ expression. Zhang et al. (2018) uncovered that TET suppressed HCC metastasis through modulating autophagy-dependent Wnt/β-catenin and metastatic tumour antigen 1 signalling.

To some extent, the discovery of Sora improved the long-term survival of HCC patients at an advanced stage. However, HCC patients treated with Sora were prone to drug resistance and adverse effects (Kudo 2019). Hence, developing novel therapeutic agents to overcome Sora resistance is imperative. In the current study, we found that TET increased the sensitivity of HCC cells to Sora by inducing apoptosis. TET has been reported as a sensitizing agent to improve the treatment efficiency of drugs in several types of malignancies (Liao et al. 2019). Jiang and Hou (2020) revealed that TET enhanced paclitaxel sensitivity in ovarian cancer *via* inhibiting the β-catenin/c-Myc/Cyclin D1 signalling pathway (Jiang and Hou 2020). Sato et al. (2019) discovered that TET increased the sensitivity of lung adenocarcinoma cells to gefitinib, possibly, *via* lysosomal inhibition. Lu et al. (2017) found that TET reversed MDR of osteosarcoma cells to paclitaxel by suppressing Pgp overexpression *via* repressing NF-κB signalling. A previous study by Wan et al. (2013) clarified that the antitumor activity of Sora plus TET might be ascribed to the induction of the intrinsic apoptosis pathway by ROS/Akt signalling. However, our study applied the network pharmacology method to comprehensively predict the possible targets, biological functions, and pathways of TET against HCC. Then, we performed a series of experiments including colony formation, cell cycle and apoptosis to elucidate the antitumor roles and mechanisms of TET *in vitro* and *in vivo* in detail.

The PI3K/AKT signalling is one of the most important intracellular pathways and is always activated in cancer, contributing to tumour progression and resistance to anticancer therapies (Martini et al. 2014; LoRusso 2016). Growing evidence suggests that natural compounds can exert antitumor effects and lower chemoresistance in HCC *via* regulating PI3K/AKT signalling (Jing et al. 2019; Liu et al. 2019; Kim et al. 2021). In the present study, the PI3K/AKT/mTOR signalling pathway was inhibited by TET or Sora, especially in the combination treatment group. Moreover, TET-induced proliferation inhibition and apoptosis were reversed by 740Y-P. More importantly, TET-mediated chemosensitivity of Sora in HCC cells was also weakened in the presence of 740Y-P. These data suggested that TET exerted anticancer activity and enhanced Sora sensitivity in HCC by inactivating the PI3K/Akt/mTOR signalling. Due to the limitation in time and finances, the multi-targets and multi-pathways of TET against HCC are not allowed to be experimentally validated one by one.

Conclusions

The pharmacological mechanism of TET against HCC was investigated by using network pharmacology followed by experimental validation (Figure 9). TET inhibited cell proliferation and cell cycle progression, induced apoptosis, and increased Sora sensitivity. Mechanistically, the influence of TET on HCC progression and Sora sensitivity was mediated by the inactivation of the PI3K/AKT/mTOR signalling pathway. Our findings highlight the potential of exploiting TET as an anticancer and chemosensitizer in the clinical treatment of HCC. Our study also confirmed the significance of network pharmacology in target screening and pathway prediction of drugs and diseases.

Disclosure statement

All authors have no conflict of interests to declare.

Author contributions

Biao Niu and Guoyong Chen are responsible for the concept design and draft writing. Biao Niu, Sidong Wei, and Jianjun Sun performed network pharmacology analysis and experimental validation. Biao Niu, Huibo Zhao, and Bing Wang are in charge of acquiring, interpreting, and analyzing data. All authors reviewed and approved the final manuscript.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. U2004124).

References

Bhagya N, Chandrashekar KR. 2016. Tetrandrine–a molecule of wide bioactivity. Phytochemistry. 125:5–13.
Dutta S, Mahalanobish S, Saha S, Ghosh S, Sil PC. 2019. Natural products: an upcoming therapeutic approach to cancer. Food Chem Toxicol. 128: 240–255.
Gan G, Shi Z, Shangguan C, Zhang J, Yuan Y, Chen L, Liu W, Li B, Meng S, Xiong W, et al. 2021. The kynurenine derivative 3-HAA sensitizes hepatocellular carcinoma to sorafenib by upregulating phosphatases. Theranostics. 11(12):6006–6018.
Gong C, Ai J, Fan Y, Gao J, Liu W, Feng Q, Liao W, Wu L. 2019. NCAPG promotes the proliferation of hepatocellular carcinoma through PI3K/AKT signaling. Onco Targets Ther. 12:8537–8552.
Grandhi MS, Kim AK, Ronnekleiv-Kelly SM, Kamel IR, Ghasebeh MA, Pawlik TM. 2016. Hepatocellular carcinoma: from diagnosis to treatment. Surg Oncol. 25(2):74–85.
Hu B, An HM, Wang SS, Chen JJ, Xu L. 2016. Preventive and therapeutic effects of Chinese herbal compounds against hepatocellular carcinoma. Molecules. 21(2):142.
Janku F, Yap TA, Meric-Bernstam F. 2018. Targeting the PI3K pathway in cancer: are we making headway? Nat Rev Clin Oncol. 15(5):273–291.
Jiang L, Hou R. 2020. Tetrandrine reverses paclitaxel resistance in human ovarian cancer via inducing apoptosis, cell cycle arrest through β-catenin pathway. Onco Targets Ther. 13:3631–3639.
Jing W, Shuo L, Yingru X, Min M, Runpeng Z, Jun X, Dong H. 2019. Artesunate promotes sensitivity to sorafenib in hepatocellular carcinoma. Biochem Biophys Res Commun. 519(1):41–45.
Keating GM. 2017. Sorafenib: a review in hepatocellular carcinoma. Target Oncol. 12(2):243–253.
Kim SY, Hwangbo H, Kim MY, Ji SY, Lee H, Kim GY, Kwon CY, Leem SH, Hong SH, Cheong J, et al. 2021. Coptisine induces autophagic cell death through down-regulation of PI3K/Akt/mTOR signaling pathway and up-regulation of ROS-mediated mitochondrial dysfunction in hepatocellular carcinoma Hep3B cells. Arch Biochem Biophys. 697:108688.
Kudo M. 2019. Targeted and immune therapies for hepatocellular carcinoma: Predictions for 2019 and beyond. World J Gastroenterol. 25(7):789–807.
Liao D, Zhang W, Gupta P, Lei ZN, Wang JQ, Cai CY, Vera AA, Zhang L, Chen ZS, Yang DH. 2019. Tetrandrine interaction with ABCB1 reverses multidrug resistance in cancer cells through competition with anti-cancer drugs followed by downregulation of ABCB1 expression. Molecules. 24(23):4383.
Liu C, Gong K, Mao X, Li W. 2011. Tetrandrine induces apoptosis by activating reactive oxygen species and repressing Akt activity in human hepatocellular carcinoma. Int J Cancer. 129(6):1519–1531.
Liu T, Liu X, Li W. 2016. Tetrandrine, a Chinese plant-derived alkaloid, is a potential candidate for cancer chemotherapy. Oncotarget. 7(26): 40800–40815.
Liu W, Li S, Qu Z, Luo Y, Chen R, Wei S, Yang X, Wang Q. 2019. Betulinic acid induces autophagy-mediated apoptosis through suppression of the PI3K/AKT/mTOR signaling pathway and inhibits hepatocellular carcinoma. Am J Transl Res. 11(11):6952–6964.
LoRusso PM. 2016. Inhibition of the PI3K/AKT/mTOR pathway in solid tumors. J Clin Oncol. 34(31):3803–3815.
Lou JS, Yao P, Tsim KWK. 2018. Cancer treatment by using traditional Chinese medicine: probing active compounds in anti-multidrug resistance during drug therapy. Curr Med Chem. 25(38):5128–5141.
Lu Y, Li F, Xu T, Sun J. 2017. Tetrandrine prevents multidrug resistance in the osteosarcoma cell line, U-2OS, by preventing Pgp overexpression through the inhibition of NF-κB signaling. Int J Mol Med. 39(4): 993–1000.
Luan F, He X, Zeng N. 2020. Tetrandrine: a review of its anticancer potentials, clinical settings, pharmacokinetics and drug delivery systems. J Pharm Pharmacol. 72(11):1491–1512.
Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. 2014. PI3K/AKT signaling pathway and cancer: an updated review. Ann Med. 46(6): 372–383.
McGlynn KA, Petrick JL, El-Serag HB. 2021. Epidemiology of hepatocellular carcinoma. Hepatology. 73(S1):4–13.
Petrick JL, Florio AA, Znaor A, Ruggieri D, Laversanne M, Alvarez CS, Ferlay J, Valery PC, Bray F, McGlynn KA. 2020. International trends in hepatocellular carcinoma incidence, 1978–2012. Int J Cancer. 147(2): 317–330.
Poornima P, Kumar JD, Zhao Q, Blunder M, Efferth T. 2016. Network pharmacology of cancer: From understanding of complex interactomes to the design of multi-target specific therapeutics from nature. Pharmacol Res. 111:290–302.
Rawat D, Shrivastava S, Naik RA, Chhonker SK, Mehrotra A, Koiri RK. 2018. An overview of natural plant products in the treatment of hepatocellular carcinoma. Anticancer Agents Med Chem. 18(13):1838–1859.
Sato E, Ohta S, Kawakami K, Ikeda M, Takahashi T, Kobayashi S, Nomura M. 2019. Tetrandrine increases the sensitivity of human lung adenocarcinoma PC14 cells to gefitinib by lysosomal inhibition. Anticancer Res. 39(12):6585–6593.
Siegel RL, Miller KD, Jemal A. 2020. Cancer statistics, 2020. CA A Cancer J Clin. 70(1):7–30.
Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. 2021. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 71(3):209–249.
Wan J, Liu T, Mei L, Li J, Gong K, Yu C, Li W. 2013. Synergistic antitumour activity of sorafenib in combination with tetrandrine is mediated by reactive oxygen species (ROS)/Akt signaling. Br J Cancer. 109(2):342–350.
Wang CH, Yang JM, Guo YB, Shen J, Pei XH. 2020. Anticancer activity of tetrandrine by inducing apoptosis in human breast cancer cell line MDA-MB-231 *in vivo*. Evid Based Complement Alternat Med. 2020:6823520.
Wang J, Yao Z, Lai X, Bao H, Li Y, Li S, Chang L, Zhang G. 2020. Tetrandrine sensitizes nasopharyngeal carcinoma cells to irradiation by inducing autophagy and inhibiting MEK/ERK pathway. Cancer Med. 9(19):7268–7278.
Wu S, Chueh F, Chou Y, Ma Y, Peng S, Lin C, Liao C, Chen P, Hsia T, Lien J. 2020. Tetrandrine inhibits cell migration and invasion in human nasopharyngeal carcinoma NPC-TW 039 cells through inhibiting MAPK and RhoA signaling pathways. J Food Biochem. 44(10):e13387.
Xiang Y, Guo Z, Zhu P, Chen J, Huang Y. 2019. Traditional Chinese medicine as a cancer treatment: modern perspectives of ancient but advanced science. Cancer Med. 8(5):1958–1975.
Yang J, Zhang L, Jiang Z, Ge C, Zhao F, Jiang J, Tian H, Chen T, Xie H, Cui Y, et al. 2019. TCF12 promotes the tumorigenesis and metastasis of hepatocellular carcinoma via upregulation of CXCR4 expression. Theranostics. 9(20):5810–5827.
Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. 2019. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol. 16(10):589–604.
Yu VW, Ho WS. 2013. Tetrandrine inhibits hepatocellular carcinoma cell growth through the caspase pathway and G2/M phase. Oncol Rep. 29(6): 2205–2210.
Zhang W, Huai Y, Miao Z, Qian A, Wang Y. 2019. Systems pharmacology for investigation of the mechanisms of action of traditional Chinese medicine in drug discovery. Front Pharmacol. 10:743.
Zhang Z, Liu T, Yu M, Li K, Li W. 2018. The plant alkaloid tetrandrine inhibits metastasis via autophagy-dependent Wnt/β-catenin and metastatic tumor antigen 1 signaling in human liver cancer cells. J Exp Clin Cancer Res. 37(1):7.
Zhao Q, Jia X, Zhang Y, Dong Y, Lei Y, Tan X, Williamson RA, Wang A, Zhang D, Ma J. 2019. Tetrandrine induces apoptosis in human neuroblastoma through regulating the Hippo/YAP signaling pathway. Biochem Biophys Res Commun. 513(4):846–851.
Zhao XM, Hu WX, Wu ZF, Chen YX, Zeng ZC. 2018. Tetrandrine enhances radiosensitization in human hepatocellular carcinoma cell lines. Radiat Res. 190(4):385–395.
Zhou Y, Mu L, Liu X, Li Q, Ding L, Chen H, Hu Y, Li F, Sun W, He B, et al. 2021. Tetrandrine inhibits proliferation of colon cancer cells by BMP9/ PTEN/ PI3K/AKT signaling. Genes Dis. 8(3):373–383.