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

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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 (IC50 = 22.5 μM) and PLC8024 (IC50 = 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 mm3 and 151.5 ± 25.8 mg compared with Sora (510.6 ± 48.2 mm3 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.

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, anti-diabetic, 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 reduces cell 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 signalling; cell cycle; apoptosis; chemosensitivity.
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.schdd.com), SwissTargetPrediction (https://www.swisstargetprediction.ch/), TCMSP (https://old.tcmsp.e.com/tcmsp.php), and PharmMapper (http://www.lilab-eust.cn/pharmmapper/submitfile.html). HCC-related targets were collected by searching three databases including TTD (http://db.idr-lab.net/ttp/), 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 cells 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% CO₂.

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 \( 5 \times 10^3 \) 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 10μL 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 (IC₅₀) 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 \( 5 \times 10^2 \) 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 × 10⁶) 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 = (Length × Width²)/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 specimens 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 PharmMapper, 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.
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 IC₅₀ 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,
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, 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 ± 1.7% in SMMC7721, 75.5 ± 1.5 vs. 60.8 ± 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 ± 0.56% in SMMC7721, 39.87 ± 3.10 vs. 18.43 ± 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.
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
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.

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.

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).

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