Metabolomics and integrated network pharmacology analysis reveal SNKAF decoction Suppresses Cell Proliferation and Induced Cell Apoptisis in Hepatocellular Carcinoma via PI3K/Akt/P53/FoxO Signaling Axis

Wei Guo
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital
Xiaohui Yao
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Siyuan Lan
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Chi Zhang
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Hanhan Li
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital
Zhuangzhong Chen
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital
Ling Yu
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital
Guanxian Liu
Huizhou Municipal central hospital
Yuan Lin
Sun Yat-sen University First Affiliated Hospital
Shan Liu
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Hanrui Chen (chenhanrui1689@gzucm.edu.cn)
Guangzhou University of Traditional Chinese Medicine First Affiliated Hospital https://orcid.org/0000-0002-0058-6609

Research Article

Keywords: hepatocellular carcinoma, network pharmacology, metabolomic analysis, SNKAF decoction, PI3K/Akt/P53/FoxO signaling pathway axis

Posted Date: February 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1305072/v1

Metabolomics and integrated network pharmacology analysis reveal SNKAF decoction Suppresses Cell Proliferation and Induced Cell Apoptisis in Hepatocellular Carcinoma via PI3K/Akt/P53/FoxO Signaling Axis

Wei Guo[1+], Xiaohui Yao[2+],Siyuan Lan[2], Chi Zhang[1], Hanhan Li[1], Zhuangzhong Chen[1], Ling Yu[1], Guanxian Liu[3], Yuan Lin[4*], Shan Liu[1*],Hanrui Chen[1*]

1. The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou University of Chinese Medicine. Guangzhou. Guangdong.P.R China:*510006
2.The Research Center for Integrative Medicine, School of Basic Medical Sciences, Guangzhou University of Chinese Medicine. Guangzhou. Guangdong.P.R China:*510006
3.Department of Nephrology, Huizhou Municipal Central Hospital, Huizhou, Guangdong.P.R China:* 516001
4.Department of Pathology, the First Affiliated Hospital of Sun Yat sen University, Guangzhou. Guangdong.P.R China:*510080

+: The authors contributed equally to this work.

*： Corresponding author.

E-mail address: chenhanrui1689@gzucm.edu.cn (Hanrui Chen); lliushan@gzucm.edu.cn (Shan Liu);Yuan Lin(liny36@mail.sysu.edu.cn)

ABSTRACT

Background： Since there is no comprehensive method on the treatment of HCC, R&D still focused on the systemic therapies about drug As alternative medicine rises, new measures for post HCC treatment have been offered gradually. SNKAF decoction, a classic Chinese herbal prescription, has been smoothly treated liver cancer in the clinic. Nevertheless, there is no research on the core active component and target of SNKAF decoction. Here, the possible active ingredients related to HCC in the SNKAF decoction have been examined.

Methods： Mouse models were established to measure the anticancer effect of SNKAF decoction on HCC. Further we investigated the SNKAF decoction on inhibiting the hepatoma cells proliferation by cell viability, cloning and invasion assays in vitro, also the induced apoptosis effects on human HCCLM3 and MHCC97H cells by Western blotting, TUNEL/Hoechst staining and flow cytometric. Secondly, the SNKAF's components were collected from the TCMSP database and TCM@Taiwan database. The HCC-related genes were obtained from GeneCards, and the protein-protein interaction (PPI) data of SNKAF's targets and HCC genes were obtained from the String database. After that, the DAVID platform was applied for Gene Ontology (GO) enrichment analysis and KEGG pathway enrichment analysis. Metabolomic analysis was used to identify the potential genes and pathways in hepatocellular carcinoma treated with SNKAF decoction. Then, the expression of PI3K、Akt、P53、FoxO proteins of potential signal pathway were detected by Western blot.

Results: The animal experiments showed that SNKAF decoction can inhibited tumor growth ($P < 0.05$)and induced no weight loss on the body weight of mice during the period. In vitro data showed that HCCLM3 and MHCC97H cell proliferation was inhibited by SNKAF serum in a time- and concentration dependent manner, the ability of cell migration and invasion was also significantly inhibited when the SNKAF serum concentration were ranged 5-20%, also the cells accumulated in S and G2/M Phase and apoptosis was increased in both cell lines by SNKAF serum. Based on the experimental study in vitro, the SNKAF serum at concentration of 10% showed good biological activity so as to facilitate further deeper research. The network pharmacology analysis results

identified 196 candidate compounds and 2537 potential targets were yielded by SFI and they shared 234 targets associated with HCC, further combined analysis with metabonomics showed that 217 target genes overlapped. The core target genes include BCL2, MCL1, Myc, PTEN, gsk3b, CASP9, CREB1, MDM2, pt53 and CCND1, cancer-associated pathways greatly took part in the mechanisms of SNKAF including p53, FoxO, phosphoinositide 3-kinase (PI3K)/Akt signaling pathways which closely related to tumor include apoptosis, cell cycle. In addition, west bolting verified that 10% SNKAF serum had significant effect on the main proteins of PI3K/Akt/P53/FoxO signaling pathway in both cell lines.
Conclusion: SNKAF decoction-containing serum inhibited HCCLM3 and MHCC97H cell proliferation, migration, invasion, induced apoptosis, and regulated the expression of tumor-related proteins. we confirmed that SNKAF decoction can promise alternative treatments for HCC patients.

Keywords: hepatocellular carcinoma; network pharmacology; metabolomic analysis; SNKAF decoction; PI3K/Akt/P53/FoxO signaling pathway axis

Introduction

As the main form of primary malignancy of the liver, hepatocellular Carcinoma (HCC) mostly originates from hepatocytes in above 80% of the cases. HCC is the third of most common cancerrelated deaths worldwide [1] . As an invasive disease often typified by the diagnosis, there is still very poort prognosis for HCC [2], with median overall survival (OS) of 5 months varying from 0 to 13 months[3]. As medical techniques are developed, the survival rate of patients with HCC grew generally, and especially in Asian population[4,5]. Because of HCC is insidious and the early symptoms are atypical or difficult to diagnose. The main curative treatment measures take part in orthotopic liver transplantation (OLT) and surgical liver resections (LR), but only about 15% of patients are suitable for surgical resection[6]. In addition, the systemic chemotherapy was one of the another effective treatment [7-9]. However, the great majority of synthesized chemotherapeutic agents have serious adverse effects[10]. Since there is no comprehensive method on the treatment of HCC, research and development still focused on the systemic therapies about drug. As alternative medicine rises, new measures for post HCC treatment have been offered gradually, particularly in developing countries, where patient consider cheap and available Chinese herbal medicines as a significant option[11]. To be specific, TCM has been extensively adopted as a pool filed with plentiful new therapeutic agents due to its beneficial effects, accessible availability, and restricted adverse effects, and have researched that Chinese medicinal formulas (CMFs) could improving the HCC patients with approximately 1-year and 2-year survival time[12,13].

Sinikangai fang（SNKAF) is prescribed traditionally for preventing and treating cancers that putatively works by decreasing the toxicity of radiotherapy and chemotherapy in tumor treatment and enhancing immunity in patients ,and has been prescribed for clinic in the first affiliated hospital of Guangdong university of Traditional Chinese Medicine to prevent and treat liver cancer. The SNKAF formula is mainly composed of Hedyotis diffusa Willd (Bai Hua She She Cao), Eupolyphaga sinensis Walker (Tu Bie Chong), Scutellaria barbataD. Don (Ban Zhi Lian), Solanum nigrum L.(Long Kui), Akebia quinata(Ba Yue Zha), Bupleurum chinense DC. (Chai Hu), Paeonia lactiflora Pall. (Bai shao), Citrus aurantium L.(Zhi Shi), Glycyrrhiza uralensis Fisch.(Gan Cao), Codonopsis pilosula (Franch.) Nannf. (Dang Sheng), Atractylodes macrocephala Koidz. (Bai Zhu), Coix lacryma-jobi Lvar. mayuen(Roman.) Stapf (Yi Yi Ren), Poria cocos(Schw.)Wolf(Fu Ling), Prunus persica (L.) Batsch (Tao Ren), Cremastra appendiculata(D. Don)Makino (Shan Ci Gu). Cancer development *via* cell cycle arrest, apoptosis induction, and immune control can be inhibited by the major compunds in the formula[14-28]. Nevertheless, due to the multi-target and multi-substance natures of this formula, it is diffcult to dig its underlying mechanisms. With the spring of genomics, proteomics, transcriptomics, metabolomics, and serum pharmacokinetics [29-31]. It coincides with the research for TCM decoction, which needs multidisciplinary cooperation and complicated analytical steps[32]. it is a complete drug-target interaction network and recognition of

the key molecules and pathways related. Besides, the increase in effectiveness is realized by cross referencing with a disease target database, to illustrate how formulas can involve in the core targets to boost the appearance and growth of disease [33,34].
Therefore, the anti-cancer effects of SNKAF on HCC and its mechanisms in vitro and vivo were assessed, and to understand the therapeutic function of SNKAF, the principles of tumor metabolism and biological physiology were adopted to examine the anti-profireration as well as enhance apoptosis of cancer cells after admission SNFAF in HCC. According to the researches, the integration strategy of network biology and multidirectional pharmacology is conducive to expanding the available drug target space especially in the field of research and development of Chinese medicine formulae.

Methods

SNKAF preparation

The proportion of all medicines in SNKAF is displayed in Table.1. On basis of the traditional decoction approach, all herbs were first soaked in distilled water for 30 min, heated to 100°C, decocted twice, then filtered to eliminate the dregs with gauze. The concentrated solution was 100mL each, cooled and kept at 4°C before combination. (All medicines were from the KangMei Pharmaceutical Co., Ltd, Guang zhou, China, and recognized in the TCM pharmacy of the First Affiliated Hospital of Guangzhou University of TCM).

SNKAF serum

Guangdong Medical Experimental Animal Center provided healthy adult Sprague-Dawley (SD) rats (weight, 150-170 g), and the rats were distributed to control group and SNKAF group (n=15). Control group was provided with a gavage of saline, and SNKAF group received SNKAF decotion. According to the pharmacological experimental methodology (4th Edition), the equal dose proportion converted by body surface area between human (70kg) and mouse (0.020kg) is 0.018. The adult human dose of 272 g/70 kg was transformed into the rat dose as = 272 g× 0.018/0.16 kg = 30.6g/kg. The rats of group B were treated with SNKAF at a dose of 5.53g/kg. Animal Ethics Committee at Guangzhou University of Chinese Medicine approved the animal experiments. Rats were dosed twice a day for 7 days. After taking blood from the aorta 2 h after the last gavage, the serum was isolated and passed through a 0.22 um filter, aseptically aliquoted and kept at -20°C for later application.

Hepatocellular carcinoma Xenotransplantation Model

Guangdong Medical Laboratory Animal Center offered four-week-old BALB/c nude mice. MHCC-97H cells at a density of 2 × 106/ul were mixed with Matrigel (Corning, New York, USA), and were injected subcutaneously into the right front leg of nude mice. When the tumor size was 80–100 mm3, the mice were assigned into five groups (n = 6) randomly , and treated with saline (model group), SNKAF lower dosage (15.3 g/kg), SNKAF medium dosage (30.6 g/kg), SNKAF high dosage (61.2 g/kg) and Sorafenib respectively. Sorafenib received intraperitoneal injection at 20 mg/kg every other day, and SNKAF was provided with oral gavage at different dosages every day. The mice were examined every 2 days, and all mice were sacrificed by CO2 quickly without suffering, and the dissection of tumours was made at the end point. Animal Ethics Committee at Guangzhou University of Chinese Medicine approved all the animal experiments.

Hematoxylin & Eosin Staining and Immunohistochemistry

The embedding of formalin-fixed tumor tissues was made in paraffin for immunohistochemistry exploration. Next, the treatment of slices with xylene and various concentrations of ethanol was made gradually, followed by immersion in distilled water. Tissue lesions are identified with H&E staining. For immunohistochemistry exploration, the treatment of slides with 3% hydrogen peroxide was conducted to block endogenous peroxidase activity, next to incubation with 10% goat serum,

then the overnight blockage of slides was conducted with anti-Ki67 antibody (Abcam, Cambridge, USA) at 4°C, and next incubated with the secondary antibody. Finally, DAB detection system (Dako A/S, Glostrup, Denmark) was adopted as chromogenic agents on basis of the manufacturer's guidance.

Cell lines and cell cultures

The human liver cancer cell lines HCCLM3 were purchased from the Shanghai Zhong Qiao Zhou Biotechnology Co.,Ltd. MHCC97H were a gift from the Institute of tropical medicine of Guangzhou University of TCM. The culture of cells was made at 37 °C, 5% CO2, followed by saturation of humidity in Dulbecco's altered Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 IU/mL streptomycin (all Hyclone, Life Sciences, Logan, UT, USA).

Cell Viability Assay and Colony Formation Assay

The human liver cancer cell lines HCCLM3 and MHCC97H cell viability were examinated by CCK-8 assays. The plating of cells was made in 96-well plates at a density of 5000 cells/well, next to adherence with incubation overnight. The treatment of cells was made with various concentrations of SNKAF serum or Sorafenib with 72 h. Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) was adopted to measure cell viabilities on basis of the manufacturer's instruction. Afterwards, a microplate reader (PerkinElmer EnSpire, Singapore) was adopted to measure the optical density OD value at 450 nm.

For colony formation investigation, the culture of cells was made in 6-well plates at a density of 5 × 103 cells per well. After cell attachment, SNKAF serum or Sorafenib was put to the wells for 24 h, followed by cultivation with fresh complete culture medium for another two weeks. The fixing of generated colonies was conducted in 4% paraformaldehyde, followed by staining with crystallization purple, photographing and counting under a microscope.

Wound Healing Assay and Transwell Invasive Assay

In terms of wound healing assays, the seeding of 4 × 105 cells was made onto 6-well plates for over 90% confluency, followed by scratch with a 1-ml pipette tip. Next, the recording of cells was made at 0 and 48 h after initiating the wound healing. Image J software (National Institute of Mental Health, Bethesda, Maryland, USA) was adopted to analyze the healing distance and healing region.

For transwell invasive assays, the coating 8-um transwell chambers was made with Matrigel, followed by placement into 24-well plates before experiment. Later, the addition of upper chambers with cells was made at a density of 1.5 × 105 according to the suggested treatment, whereas complete DMEM medium with 10% FBS filled the lower chamber for 48 h. In the end, a cotton swab was used to erase the upper cells, while after being fixed with 4% formaldehyde, the 30-minute dying of cells invaded into the bottom of chamber with 0.5% hematoxylin solution was made, next to photographing for detection.

Flow Cytometry Analysis

For the drug efflux assay, the placement of cells was made onto 6-well plates at a density of 3 × 105 cells/well, followed by treatment with various doses of SNKAF serum or Sorafenib for 48 h. For cell cycle exploration, the overnight fixing of cells was conducted in ice-cold 70% ethanol at 20°C. Next, phosphate-buffered saline was adopted to wash cells, followed by staining with 50 mg/mL propidium iodide (BD Biosciences, San Jose, CA, United States). For apoptosis exploration, the Annexin V-FITC Apoptosis Staining/Detection Kit (BD Biosciences) was adopted to stain the cells. All flow cytometry analyses were made with BD Accuri C5 or LSRFortessa and FlowJo software.

Hoechst 33258 and TUNEL Staining

Immunofluorescence Analysis the HCC apoptosis after admission SNKAF serum. The seeding of growth phase cells into 6-well plates was performed at a density of 1x105 cells a certain time. The incubation of cells was made with various concentrations of SNKAF or Sorafenib. After 48-hour

incubation, the cells were washed with PBS and fixed by 4% polyformaldehyde. After washing the cells with PBS buffer, Hoechst 33258 solution (10 ug/mL) was adopted to stain th cells after washing twice, followed by 20-minute incubation in dark to obtain the stability samples. The InSitu Cell Death Detection Kit (Roche) was adopted for TUNEL analysis according to the manufacturer's protocols. In the end, anti-fluorescence quenching mounting solution was employed to mount cells before putting them under observation with a confocal microscope (Zeiss LSM800).

Metabolomic profiling

After collection, the 10-minute centrifugal of plasma samples was made at 1000 rpm. The thawing and equilibrium of all samples were performed at 4 °C, and then 1 mL of acetonitrile: methanol: ddH2O mixed solution (2:2:1, v/v/v) was put to the plasma samples, mixed and centrifuged at 4°C for 10 min at 12 000 rpm. Next, the transfer and evaporation of 850~900 uL of thesupernatant were made to dryness. After being vortex-mixed with 300 uL of 2-chlorobenzalanine solution (4 ppm), the dissolved samples were centrifuged. In the end, for the prepared samples for the LC-MS analysis, the supernatant was screened through a 0.22 um membrane.

Chromatographic conditions： the plasma samples were made in the Thermo Scientific Vanquish UHPLC-Q Exactive system and Hyperil Gold column(C18) column (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, USA) which the temperature of the column was kept at 40 °C . Solvent A was water (including 0.1% formic acid), while solvent B was acetonitrile/isopropanol (1/1) (including 0.1% formic acid). The flow rate was set at 0.20 ml/min, and each sample was injected within volume of 20 ul. The gradient elution program was shown below: 0 min 2% B, 0–2 min 2% B, 2–12 min 98% B, 12–14 min 98% B, 14.0–14.1 min 2% B, 14.1–17 min 2% B.

The ESI-MSn experiments were conducted on a thermo Q precision mass spectrometer to examine the stability of the instrument. and the spray voltages were 3.2kV,for the positive and negative modes. Sheath gas flow rate were sest at 10~40arb,respectively. The capillary Tempwas set at 320°C. An HCD scan was carried out to make data dependent acquisition (DDA) MS/MS experiments, several unnecessary data in the MS/MS spectra was eliminated with dynamic exclusion.

Etablishment of an Ingredient-Target-Liver Cancer Network

The compounds information of SNKAF decoction were gathered from Traditional Chinese Medicine Systems, and the database platform were used included the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, *http://lsp.nwu.edu.cn /index.php*), Traditional Chinese medicine ingredient database (TCMID, *http://119.3.41.228:8000/*), A Bioinformatics Analysis Tool for Molecular mechANism of Traditional Chinese Medicine (BATMAN-TCM, *http://bionet.ncpsb.org/batman-tcm/*), and the TCM database@Taiwan (TCM@Taiwan, *http://tcm.cmu.edu.tw/zh-tw/*). The screening of ingredients was made on basis of drug likeness (DL) and OB values, and the retaining of ingredients was realized if DOB ≥ 30%, DL ≥ 0.18, and Caco2 > 0, a standard indicated by the TCMSP database.

And then identify the potential molecular targets of each recognized compounds though the Genecards database, BATMAN-TCM (*https://www.genecards.org/*), and the STRING database (*https://string-db.org/*) . The ingredient–target networks were applied via Cytoscape software (version 3.2.1). The subjection of target genes to the Database for Annotation, Visualization, and Integrated Discovery (DAID, *https://david.ncifcrf.gov/*) and the Comparative Toxic Genetics Database (CTD, *http://ctdbase.org/*) was made to implement GO and KEGG enrichment for functional enrichment analysis, applying the clusterProfiler software package for R3.5.3 to evaluate the hidden biological functions, and p value < 0.05 suggested greater enrichment[47].

Data analysis

Principal Component Analysis (PCA) was adopted for quality control analysis; the classifications for different groups were observed with Partial Least Squares Discrimination Analysis (PLS-DA) and Orthogonal Partial Least Squares Discrimination Analysis (OPLS-DA). Ions with variable importance (VIP) > 1 and P-value < 0.05 were considered as differentiated metabolite ions. In the

advanced data exploration, the heatmap analysis and KEGG enrichment exploration were made via the Majorbio Cloud Platform. Pathway analysis was made with Metabo Analyst.

Statistical analysis

One-way analysis from the GraphPad Prism v6.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used to compare different groups. Data were shown as the mean ± SD of at least three repeated experiments. It was thought that **P*-value under 0.05, ***P*-value under 0.01 and ****P*-value under 0.001 are statistically significant.

Results

SNFAF Suppresses Growth in vivo of liver Cancer Xenotransplantation Model

While SNKAF has been empirically adopted as an adjuvant in clinically treating hepatocarcinoma, evidence currently available illustrating its mechanisms is limited. The anticancer-like activity of SNKAF was assessed in nude mice with xenografted tumors. Firstly, MHCC-97H cells were subcutaneously injected into the mice, and then randomly assigned to five groups: (1) Model (normal saline); (2) SNKAF-Low; (3) SNKAF-Medium; (4) SNKAF-High and (5) Sorafenib, after administration of SNKAF decotion or Sorafenib, the mice were sacrificed for tumor tissue collection, SNKAF significantly inhibited tumor volume (Figure 1A and B), and the average tumor volume was greatly smaller from Day 6 to the end of the research in the SNKAF-High-group , SNKAF-Medium and low group exrted significantly inhinitored from day 8 by comparing with the model group (Figure 1C). During the whole experiment, SNKAF treatment induced no weight loss on the body weight of mice, and a obviously toxic after Sorafenib administration (Figure 1D). H&E pathological staining (Fig. 1E) showed that Sorafenib treatment brought extensive cell death, according to evidence by smeared cell morphology and the extinction of nuclear staining. And treatment with SNKAF also caused cell death. Ki67 is an important cell nuclear proliferation marker [35], the tumor cell proliferation was greatly impaired by the SNKAF or Sorafenib treatment (Fig. 1F). Together, these findings confirmed that SNKAF inhibited tumor growth in human liver tumor-bearing xenograft mice.

SNKAF Serum Exerts Anti-Proliferation, Migrative and Invasive Potentia Effects on HCCs

Based on SNKFA had significantly inhibit influence on liver cancer growth in vivo. The anti-cancer effect of SNKAF on cancer cell lines including HCCLM3 and MHCC97H was assessed. The viability of both hepatoma cell lines were inhibited in a concentration- and time-dependent manner by cck-8 assay (figure 2A-B). Figure 1A showed the IC50 of SNKAF serum or Sorafenib for the suggested cell lines. To be specific, the IC50 values of SNKAF at 48 h for HCCLM3 and MHCC97H were 13.9%, and 12.3%, and Sorafenib were 6.5 and 5.5 uM, respectively. In MHCC97H cells, the cells viability were inhibited at three SNKAF serum groups by comparing with the blank control cultures at 48 and 72 h. Rat blank serum at 20% was observed a markedly inhibitory effect on cell proliferation at after 48h and the concentration at 10% had no effect on cell at times 24-72h compared with the blank control group. In HCCLM3 cells, SNKAF serum at concentration of 10% and 20% had a obvious inhibiting effect by comparing with the blank control group at 48 and 72h. The rat blank serum at 20% could inhibit the cell growth at 24h, the concentration at 10% had no effect on cell at times 24-72h by comparing with the blank control group. So choose the blank serum at 10% for deeper research.

Then, a colony formation assay confirmed the inhibitory effect on HCCs.A reduced number of colonies that formed in the different group was showed in Figure 2C, SNKAF serum blocked the colony-forming capability in a dose-dependent manner in both HCCLM3 and MHCC97H cells. wound healing assays were adopted to assess the potential of SNKAF serum on the migrative capacity. According to Figure 2D, the gap widths and regions of the untreated group were narrowed more quickly by comparing with those of the SNKAF serum or Sorafenib group from 0 to 48 h. Besides, according to chamber invasive assay, the aggressive cell number was greatly decreased according to SNKAF serum treatment at concentration of 5~20% (Figure 2E).Taken together, these

data suggest that SNKAF serum could suppress HCC cell proliferation, invasive and migrative ability in vitro.

SNKAF Serum Induced Apoptosis in HCC Cells and Arrest HCC Cell Cycle at the S and G2/M Phase

In order to investigate the anticancer mechanism which SNKAF serum inhibits HCC cells proliferation,In the S phase, the cell synthesises DNA in its nucleus, duplicating a microtubule-organising structure called the centrosome to isolate DNA during the M phase. In the G2/M phase the cell growth rapidly to producing proteins and organelles, and starts reorganizing its contents in preparing for mitosis. A increase in the S with G2/M population shows a growth in the apoptotic cells. PI staining was adopted to investigate the roles of SNKAF serum in cell cycle distribution by flow cytometry analysis. According to Figure 3A, the flow cytometry results revealed that SNKAF serum could induce G2/M and S checkpoint in both liver cancer cell lines. when Sorafenib arrested the cell at G2/M and S phase were 67.44% in MHCC97H cells and 55.44% in HCCLM3 cells, and SNKAF serum with different dose induced cell cycle arrest at G2/M phase were 36.03%, 37.43%, 51.57%, compered with 29.84% in rat serum group for MHCC97H cells, and were 36.88%, 39.92%, 45.75%, compered with 30.43% in rat serum group for HCCLM3 cells.

As another significant mechanism indicated cell death caused by drugs is apoptosis[36]. The apoptotic events were detected with annexin V/PI staining. According to Figure 3B, the percent of early and late apoptotic events in HCCLM3 and MHCC97H cells was about 28.56% and 39.8%, after being exposed to Sorafini. And when SNKAF-serum at 5%-20% dosage was administrated, the percentage HCCLM3 and MHCC97H apoptotic cells was grown to about 10.4%, 12.25%,17.43%; MHCC97H apoptotic cells was increased to about 17.3%, 18.69% and 25.73 %. For further confirming the protective roles of SNKAF serum effect in HCC cells, Hoechst staining was adopted to examine apoptotic features and TUNEL staining to examine DNA fragmentation and cell death in HCCs with or without SNKAF serum treatment. As shown in Figure 3C and D, SNKAF serum greatly reduced the percentage of apoptotic cells tested by Hoechst and TUNEL staining in both HCCLM3 and MHCC97H cells. Together, these results confirmed that SNKAF serum promoted cells apoptosis in HCCs.

Network pharmacology predicted the potential target of SNKAF on HCC

The collection of the chemical ingredients of SNKAF was made in the TCMSP Databases. As a unique systems pharmacology platform of TCM, the database captures the associations among drugs, targets and diseases. Firstly, we screened candidate compounds for oral bioavailability (OB), Caco-2 permeability, and drug likeness (DL) satisfied the criterion (OB $\geq$ 30%, DL $\geq$ 0.18, and Caco-2 $\geq$ 0)in the TCMSP database, yielded 196 candidate compounds in total (Supplementary Table 1), and obtained 2537 genes extracted from the Genecard (Figure 4B), 234 of them overlapped with the HCC targets in the Venn diagram analysis (Figure 4A). For identifying the functions and mechanisms of SNKAF, GO and KEGG pathway-enrichment analysis was made on basis of the background of all human genes. For GO-term analysis, positive control of transcription from RNA polymerase II promoter, response to drug, signal transduction, negative control of apoptotic process, positive control of transcription, DNA-templated, positive control of cell proliferation, oxidation-reduction process, apoptotic process, positive control f gene expression, aging, negative control of transcription from RNA polymerase II promoter were most greatly related tp SNKAF action (Figure 4C). For KEGG pathway discussion, anti-cancer mechanisms of SNKAF might be tightly related to various intervened pathways such as the Pathways in cancer,PI3K-Akt signaling pathway,Hepatitis B, Proteoglycans in cancer, MicroRNAs in cancer, Neuroactive ligand-receptor interaction, Prostate cancer, Focal adhesion, MAPK signaling pathway, Calcium signaling pathway (Figure 4D). The filtering of pathway network for the hub genes was made in Figure 4E, the squares indicated that the pathways and the target genes taking part in the network were set by the circle.

Metabolomics analysis identified the differential metabolism-target of SNKAF in rat's plasma

To know the metabolic roles of SNKAF in HCC, this paper examined and analyzed differential metabolites in plasma samples from rat. In the basic data analysis, Figure 5 displays the basal peak intensity (BPI) chromatograms of the serum supernatants of the Control, SNKAF groups, indicating good peak shape and relatively uniform distribution under the test conditions. A 3D-PCA analysis (Figures 6A) ,PLS-DA (Figures 6B) ,Vol - cano (Figures 6C) of the Control and SNKAF groups were performed with Them EZinfo. Good isolation was realized with both groups, showing improved abnormal metabolism after the administration of SNKAF. Heat map analysis of the total 89 differential metabolites in pos- and neg- ion mode (Figures 6E), the degree of variation is labelled with various colors, every row stands for a single sample, and every column stands for a metabolite, and the more information, refer to (Supplementary Table 2). Metabolic pathway analysis according the differential metabolites by KEGG (Figures 6D), five pathways of Metabolic pathways, Biosynthesis of amino acids, Phenylalanine metabolism, ABC transporters, and Vitamin digestion and absorption were found(accroding p value =1), and the metabolites including Indole-3-acetic acid, Bilirubin, L-Ornithine, D-Proline, Glycocholic acid, Riboflavin, trans-Cinnamic acid, 4-Methylphenol, Gluconolactone, D-Glucose 6-phosphate, 2-Oxoadipic acid, Hippuric acid, the more information in Supplementary Table 3.

Integrated analysis of metabolomics and network pharmacology

For an integrated vision of the mechanisms of SNKAF against HCC, an interaction network was set up on basis of metabolomics and network pharmacology. The target genes obtained by differential metabolites intersect with the 234 differential genes obtained cross network pharmacology, 217 of them overlapped in the Venn diagram analysis (Figure 7A), The String database online service platform was adopted to perform Protein–protein interaction network analysis on the HCC targets as displayed in Figure 7B. The yellow nodes in the middle stand for the genes may exert significant effects on the genesis and progression of HCC though the MCODE plug in in Cytoscape,and there were BCL2, MCL1, MYC, PTEN,GSK3B, CASP9, CREB1, MDM2, PT53, CCND1 (Figure 3C) , The top 10 important terms in GO functional enrichment and the top 20 important KEGG pathways are displayed in Figures 7D and E. KEGG analysis ($P < 0.05$) suggested that various cancer-associated pathways greatly took part in the mechanisms of SNKAF such as apoptosis, cell cycle, p53, HIF-1, ErbB, FoxO, phosphoinositide 3-kinase (PI3K)/Akt signaling pathways. Remarkably, different targets in (PI3K)/Akt signaling can be boosted by a number of stress signals suchas DNA harm caused by the p53 signaling consequently resulting in cell cycle arrest and apoptosis[37], the cell cycle effect of the foxo signaling pathway[38], and even influencing apoptotic markers such as Bax, Bcl-2.

Validation the SNKAF as a Potential Anti-cancer Inhibitor through the PI3K/Akt/P53/FoxO Axis in HCCs

Since both metabolomics and KEGG analysis indicated that apoptosis was necessary for the anti-cancer function of SNKAF, the role of SNKAF in apoptosis flux in HCCs was assessed. According to Figure 7E, the panel displays the PI3K/AKT signaling pathway from KEGG result, the red rectangle nodes stand for the most prominent genes associated with SNKAF pharmacological actions, and those protein levels were assessed, suggesting that PI3K, Akt, FoxO3a, BAD, BCL-2,P53, western blot analysis (Figure 8) showed that, among the various treatment groups, injection of sorafini or SNFAF serum, the 10% rat serum have no obvious effect on the protein expression of the ratios of p-Fox3a/Fox3a, p-PI3K/PI3K and p-Akt/Akt, p-p53/p53 and cleaved-caspase-9, cleaved-caspase-3, bax, bcl-2 on HMCC-97H, 10% SNFAF serum and sorafini promoted the protein expression of cleaved-caspase-9, cleaved-caspase-3, bax and the ratios of P-p53/p53, while diminished protein expression of Bcl-2 and the ratios of P-Fox3a/Fox3a, p-PI3K/PI3K and p-Akt/Akt. And also the same trend was observed in HCCLM3. These results indicate that the effects of SNKAF decoction on anti-cancer of HCC were associated with the cell prolication and apoptatic

involved PI3K/Akt pathway axis with the downstream cell survival P53 signal pathway,cell cycle progression FoxO signal pathway (Figure 9).

Discussion

Currently, the world has extensively focused on TCM on treating or the preventing different diseases[39] such as cancers[40]. SNKAF decoction exerts a significant effect on enhancing the symptoms of liver patients in the clinic, but there is no clear demonstration of potential mechanism so far, particularly in terms of its active compound and the core target.Therefore, network pharmacology and metabolomics are combined with biological approaches to understand the potential mechanism of SNKAF decoction.

SNKAF formula contains 14 herbal compounds, there are more than 197 active compounds based on pharmacological research(Supplement Table 1), and the verification of whether these compounds have a pharmacodynamic effect on the body is also quite complex. Based on serum pharmacology can eliminates the interference of the physical and chemical properties of traditional Chinese medicine preparations, and also shows the last process of digesting and absorbing Chinese traditional medicine in the gastrointestinal tract. There were research on administration of Acanthopanax senticosus to mice and found 12 prototype components and 9 metabolic components, which were screened faster than 131 components in vitro[41], moreover this method reflect the real effective function of drug components affecting the body. We used the SNKAF serum as the research object to study the pharmacodynamic mechanism, there 89 different metabolic after admission SNKAF, 24 metabolic were down regulated, while 65 metabolic were up regulated. Biosynthesis of amino acids plays an important role in many research activities in cancer, such as Pro regulates cytoplasmic balance and a significant component of collage.Its biosynthetic is necessary for remodeling the tumor microenvironment and extracellular matrix to boost the reprogramming and proliferation of cancer cells. Besides, Pro can produce ATP for cell growth through the TCA cycle during catabolism. There are research on at the primary growth of HCC, amino acids originated from the degradation of proteins in liver tissue or other tissues may increase greatly, and are then taken up by amino acid transporters in liver cancer tissue to aid rapid proliferation[45]. Phenylalanine metabolismis transformed into tyrosine by the catalytic oxidation of phenylalanine hydroxylase, and tyrosine takes part in glucose and fat metabolism in the body, which are the main energy sources for cancer cells grow rapidly[42].Phenylalanine level were significant increased in hepatocarcinoma with lung metastasis, which may be related with decreased catabolism in the live [44]. Therefore, in our study, the significant difference in Biosynthesis of amino acids and Phenylalanine metabolism of the serum metabolites in SNKAF decoction. It forecasted that Biosynthesis of amino acids and Phenylalanine metabolism as the main metabolic pathways taking part in various cellular processes including cancer cell proliferation, migrate and so on.

As multi-target measures for cancer treatment attract increasing attention, there are a research strategies relying on network pharmacology combine with metabolomics to study the intricate associations between TCM formulas and the related targets and diseases, and further combined with bioinformatic analysis, to explore the disease mechanisms and intervention strategies[43,44]. By integrating metabolomics with network pharmacology, 5 core targets (BCL2, GSK3B, CASP9, MDM2, PT53) were closely related to three signaling pathways (PI3K/AKT signaling pathway, P53 signaling pathway, FoxO signaling pathway). This strategy provides a suitable method to verify the results of the two approaches. Next, the vivo assay found that SNKAF could effectively suppressed the growth and prolonged the survival of subcutaneous tumors in mice (figure 1). Hence, the molecular biology experiments in vitro were conducted to verify the anti-cancer effect on cells, like CCK-8, Invasion, Apoptosis and cell cycle assays, were used to observe the exact efficacy of SNKAF on HCC. and SNKAF exerts anti-tumor effects by accelerateing cancer cell apoptosis

(figure 3) as well as inhibiting HCC growth, progression (figure 2 and 3), migrative and invasive (figure 2).
As cell cycle and cell death signaling pathways exert significant effects on cancer growth, they act as hidden cancer therapeutic targets [55]. Really, compounds inducing cell cycle arrest and apoptosis may act as a valuable measures for cancer drug discovery. And then according the previous bioinformatic assay forecasted that SNKAF formation affected the HCC pathway via the metabolomics study with network pharmacolgy, According to the signaling pathway enrichment, these signaling pathways are closely associated with the cell cycle and death pathological changes of HCC. PI3K/AKT pathway, it exerts a significant effect on controlling the cell survival, proliferation, migration, and metabolism and apoptosis among different tumor kinds, including HCC [46–48]. When the compounds of SNKAF boosted upon the binding of the signaling molecules,it will provoking their related receptors at the surface of the cell membrane, resulting in the receptor PI3K conformational variations and the follow-up recruitment, phosphorylation, after being activated, PI3k induces the activation of a core signaling kinase AKT, which controls some downstream effector molecules like glycogen synthase kinase-3β (GSK3β)GSK3, driving protein and lipid synthesis and finally cell development[49-50]; forkhead box O (FOXO) family, participating in various cellular physiological processes and regulation cell proliferation, cell cycle [51,52]. Besides, the activated AKT may inhibit apoptosis and drives cell survival via the follow-up modulation of different target molecules including the Bcl-2 family of proteins [53–55]. and also the activation of the Akt can promote the up-regulating of MDM2, a main negative regulators of P53 that may strengthen the degradation of P53 and inhibit its phosphorylation to restrain apoptosis an modulate the cell cycle [56-57].

Conclusion
In this study, the researches indicated that SNKAF efficacy was by the synergistic role of multi-compounds, multi-targets, and multi-pathways. For verifying the molecular mechanism of SNKAF's effect on HCC, western blotting assays were made to show that SNKAF reduced the levels of phosphorylated PI3K, inhibiting the cell proliferation and recruitment in cytoplasm, further influence phosphorylated AKT,on the one hand, SNKAF decreased the levels of p-AKT, which in turn targets downstream apopotsis regulatory markers, like caspase 9, bax, bim, Bcl-XL, leading the occurrence of apoptosis in HCC cell. On the anthour hand, SNKAF regulated the progress of apopotsis through phosphorylated AKT mediated the interaction of p53 with basal transcriptional machinery components and the enhancement of the expression of genes including Bax, Therefore, the novelty of our study is the first exploration on the association with antiapoptotic signaling pathway aixs in HCC following SNKAF decotion treatment.

List of abbreviations: SNKAF, Si Ni Kang Ai Fang ; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, Gene Ontology; H&E staining, hematoxylin-eosin staining; IC50, the half maximal inhibitory concentration; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; PI, propidium iodide; PPI, protein–protein interaction; TCM, traditional Chinese medicine; , Traditional Chinese Medicine Integrated Database; TCMSP, Traditional Chinese Medicine Systems Pharmacology; TUNEL, terminal dexynucleotidyl transferase(TdT)-mediated dUTP nick end labeling.

Ethics approval and consent to participate
The experiments using nude mice were approved by institutional guidelines for the care and use of laboratory animals approved by the Animal Care and Use Committee of Guangzhou University of Chinese Medicine.

Consent for publish
Not applicable.

Availability of data and materials

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This research was supported by the Guangdong Natural Science Foundation (2021A1515011489), the Science and Technology Planning Project of Guangzhou (201904010396, 202002030376), Scientific research project of Guangdong Bureau of traditional Chinese Medicine (20212051), University Science and technology project of Guangdong Provincial Department of Education (2020KTSCX028).

Authors' contributions

Guanxie Liu,Hanrui Chen: Conceived and designed the project. Hanhan Li, Zhuangzhong Chen: Provided consultation. Wei Guo, Xiaohui Yao,Siyuan Lan: Performed animal experiments. Xiaohui Yao,Siyuan Lan, Hanhan Li: Performed in vitro experiments. Chi Zhang:Performed network pharmacology analysis. Wei Guo, Xiaohui Yao,Shan Liu:Performed Metabonomic data analysis. Guanxie Liu, Yuan Lin: Analyzed data. Wei Guo, Xiaohui Yao:Wrote the manuscript.Yuan Lin, Shan Liu,Hanrui Chen:Revised draft.All authors had approved the manuscript.

Acknowledgements

Not applicable.

References

1. Yang, J. D. et al. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat. Rev. Gastroenterol. Hepatol. 2019; 16, 589–604.
2. EASL. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol. 2018; 69(1):182–236. doi: 10.1016/j.jhep.2018.03.019
3. Sweta Soni, Puneet Pareek, Satya narayan, Amrita Rakesh, Abhilasha Abhilasha. Hepatocellular Carcinoma (HCC) in North-Western India: A Retrospective Study Focusing on Epidemiology, Risk Factors, and Survival. Journal of Gastrointestinal Cancer. 2021.;9 (22). https://doi.org/10.1007/s12029-021-00712-z.
4. Davila JA, El-Serag HB. Racial differences in survival of hepatocellular carcinoma in the United States: a population-based study. *Clin Gastroenterol Hepatol.* 2006;4(1):104-110; quiz 104-105.
5. Khalaf N, Ying J, Mittal S, et al. Natural History of untreated hepatocellular carcinoma in a US cohort and the role of cancer surveillance. *Clin Gastroenterol Hepatol.* 2017;15(2):273-281.e1.
6. Czigany Z, Schöning W, Ulmer TF, Bednarsch J, Amygdalos I, Cramer T, et al. Hypothermic oxygenated machine perfusion (HOPE) for orthotopic liver transplantation of human liver allografts from extended criteria donors (ECD) in donation after brain death (DBD): a prospective multicentre randomised controlled trial (HOPE ECD-DBD). BMJ Open. 2017; 7(10): e017558. doi: 10.1136/bmjopen-2017-017558
7. Kudo M, Finn RS, Qin S, Han K-H, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in fifirst-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018; 391(10126):1163–73. doi: 10.1016/S0140-6736(18)30207-1
8. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. Lancet. 2017; 389(10088):2492–502. doi: 10.1016/S0140- 6736(17)31046-2
9. Bruix J, Qin S, Merle P, Granito A, Huang Y-H, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017; 389(10064):56–66. doi: 10.1016/S0140-6736 (16)32453-9
10. Burkhart, R. A., Ronnekleiv-Kelly, S. M. & Pawlik, T. M. Personalized therapy in hepatocellular carcinoma: molecular markers of prognosis and therapeutic response. Surg. Oncol.2017; 26, 138–145 .

11. B. P. Gaire, “Herbal Medicine in Ischemic Stroke: Challenges and Prospective,” Chinese Journal of Integrative Medicine.2018; vol. 24, no. 4, pp. 243–246, .
12. S. W. Seto, D. Chang, A. Jenkins, A. Bensoussan, and H. Kiat, “Angiogenesis in Ischemic Stroke and Angiogenic Effects of Chinese Herbal Medicine,” Journal of Clinical Medicine.2016; vol. 5, no. 6, p. 56, .
13. Yingqi She, Qinfeng Huang, Zhen Ye, Yu Hu, Mingquan Wu, Kaihua Qin, Ailing Wei, Xin Yang, Yuyao Liu, Cuihan Zhang, and Qiaobo Ye.The Therapeutic Principle of Combined Strengthening Qi and Eliminating Pathogens in Treating Middle-Advanced Primary Liver Cancer: A Systematic Review and Meta-Analysis.Frontiers in Pharmacology. 2021; (12) : 714287.doi: 10.3389/fphar.2021.714287
14.Zhou P, Shi W, He XY,et al. Saikosaponin D: review on the antitumour effects, toxicity and pharmacokinetics. Pharm Biol. 2021; Dec;59(1),DOI：10.1080/13880209.2021.1992448.
15.Bae T, Jang J, Lee H,et al. Paeonia lactiflora root extract suppresses cancer cachexia by down-regulating muscular NF-κB signalling and muscle-specific E3 ubiquitin ligases in cancer-bearing mice. J Ethnopharmacol. 2020;Jan 10:246, DOI：10.1016/j.jep.2019.112222.
16.Li L, Zhao R, Li Y,et al. Antitumor activity of 2-[(2E)-3,7-dimethyl-2,6-octadienyl]-6-methyl-2,5-cyclohexadiene-1,4-dione isolated from the aerial part of Atractylodes macrocephala in hepatocellular carcinoma. Mol Med Rep. 2017; 16(5),DOI：10.3892/mmr.2017.7385
17. Bai R, Li W, Li Y,et al. Cytotoxicity of two water-soluble polysaccharides from Codonopsis pilosula Nannf. var. modesta (Nannf.) L.T.Shen against human hepatocellular carcinoma HepG2 cells and its mechanism. Int J Biol Macromol. 2018;120,DOI：10.1016/j.ijbiomac.2018.09.123.
18. Wang J, Liao AM, Thakur K,et al.Licochalcone B Extracted from Glycyrrhiza uralensis Fisch Induces Apoptotic Effects in Human Hepatoma Cell HepG2. J Agric Food Chem. 2019;27:67(12),DOI：10.1021/acs.jafc.9b00324.
19.Wu Y, Cheng CS, Li Q,et al. Citrus foliumThe Application of in Breast Cancer and the Mechanism of Its Main Component Nobiletin: A Systematic Review. Evid Based Complement Alternat Med. 2021;2021,DOI：10.1155/2021/2847466.
20.Chen XZ, Cao ZY, Chen TS,et al. Water extract of Hedyotis Diffusa Willd suppresses proliferation of human HepG2 cells and potentiates the anticancer efficacy of low-dose 5-fluorouracil by inhibiting the CDK2-E2F1 pathway. Oncol Rep. 2012;28(2),DOI：10.3892/or.2012.1834
21. Xie X, Shen W, Zhou Y, et al. Characterization of a polysaccharide from Eupolyphaga sinensis walker and its effective antitumor activity via lymphocyte activation.Int J Biol Macromol. 2020;01:162, DOI：10.1016/j.ijbiomac.2020.06.120.
22. Yang PW, Chen TT, Zhao WX,et al. Scutellaria barbata D.Don and Oldenlandia diffusa (Willd.) Roxb crude extracts inhibit hepatitis-B-virus-associated hepatocellular carcinoma growth through regulating circRNA expression. J Ethnopharmacol. 202;115:275, DOI：10.1016/j.jep.2021.114110.
23. Yang MY, Hsu LS, Peng CH,et al. Polyphenol-rich extracts from Solanum nigrum attenuated PKC alpha-mediated migration and invasion of hepatocellular carcinoma cells. J Agric Food Chem. 2010; 12:58(9), DOI：10.1021/jf100718b.
24. Wang H, Wang X, Li Y, et al. Structural properties and in vitro and in vivo immunomodulatory activity of an arabinofuranan from the fruits of Akebia quinata. Carbohydr Polym. 202; 15;256.
25.Du W, Zhu W, Ge W, et al. Research on the effect of spleen-invigorating and anti-swelling active ingredients in crude and processed coix seed based on Spectrum - Effects relationship combined with chemometrics. J Pharm Biomed Anal. 2021; 31:205,DOI：10.1016/j.jpba.2021.114350.
26.Liu X, Wang X, Xu X, et al. Purification, antitumor and anti-inflammation activities of an alkali-soluble and carboxymethyl polysaccharide CMP33 fromPoria cocos. Int J Biol Macromol. 2019:15:127,DOI：10.1016/j.ijbiomac.2019.01.029.
27. Lee CK, Park KK, Hwang JK, et al. The extract of Prunus persica flesh (PPFE) attenuates chemotherapy-induced hepatotoxicity in mice. Phytother Res. 2008; 22(2), DOI：10.1002/ptr.2296
28. Terada S, Motomiya T, Yoshioka K,et al.Antiallergic substance from Asarum sagittarioides and synthesis of some analogues.Chem Pharm Bull (Tokyo). 1987; 35(6), DOI：10.1248/cpb.35.2437.
29. Zhang, R., Zhu, X., Bai, H., Ning, K., Network Pharmacology Databases for Traditional Chinese Medicine: Review and Assessment. Front Pharmacol. 2019;10,:123.
30. S. Li and B. Zhang, “Traditional Chinese medicine network pharmacology: theory, methodology and application,” Chinese Journal of Natural Medicines.2013; vol. 11, no. 2, pp. 110–120, .
31.L. Shao, B. Zhang, and N. B. Zhang, “Network target for screening synergistic drug combinations with application to traditional Chinese medicine,” BMC Systems Biology. 2011; vol. 5, Suppl 1, p. S10,
32. Zhang, R.Z., Yu, S.J., Bai, H., Ning, K. TCM-Mesh: The database and analytical system for network pharmacology analysis for TCM preparations. Sci Rep; 2017; 7(1), 2821.
33. K. Yang, L. Zeng, T. Bao, Z. Long, and B. Jin. “Exploring the pharmacological mechanism of quercetin-resveratrol combination for polycystic ovary syndrome: a systematic pharmacological strategy-based research,”. Scientifific Reports. 2019; vol. 9, no. 1, p. 18420.
34. Roberts, L.D., Souza, A.L., Gerszten, R.E., Clish, C.B. Targeted metabolomics. Curr Protoc Mol Biol. 2012. Chapter 30, Unit 30 32 31-24.
35. Kun, N., Kang, L., Tingting, S., Kaikai, W., Ying, W., and Xingya, W. Anticarcinogenic effects of water extract of sporoderm-broken spores of Ganoderma lucidum on colorectal cancer in vitro and in vivo. Int. J. Oncol. 2017. 50 (5), 1541–1554. doi: 10.3892/ijo.2017.3939

36. Li, Q. Q., Wang, G., Huang, F., Banda, M., and Reed, E. (2010). Antineoplastic effect of beta-elemene on prostate cancer cells and other types of solid tumour cells. J. Pharm. Pharmacol. 2010. 62, 1018–1027. doi: 10.1111/j.2042-7158.2010. 01135.
37. Mello, S. S., and Attardi, L. D. (2017). Deciphering p53 signaling in tumor suppression. *Curr. Opin. Cell Biol.* 51, 65–72. doi: 10.1016/j.ceb.2017.11.005
38.Min Tan, Yanxiang Cheng, Xiaozhu Zhong, Dongyong Yang, Sushi Jiang, Yang Ye, Miao Ding, Guijun Guan, Dongzi Yang, Xiaomiao Zhao.LNK promotes granulosa cell apoptosis in PCOS via negatively
regulating insulin-stimulated AKT-FOXO3 pathway. AGING. 2021, Vol. 13, No. 3(4617-4633).
39. Rong-Bin Chen, Ying-Dong Yang, Kai Sun, Shan Liu, Wei Guo, Jin-Xin Zhang, Yong Li.Potential mechanism of Ziyin Tongluo Formula in the treatment of postmenopausal osteoporosis: based on network pharmacology and ovariectomized rat model. Chinese Medicine. 2021.16:88. https://doi.org/10.1186/s13020-021-00503-5
40. Ju-Min Huang, Yi-Zhong Zhang, Ze-Bo Jiang, Tu-Liang Liang, Lin-Rui Ma , Jian Wang ,Xuan-Run Wang, Xiao-Qing Fan, Jie Huang, Ying Xie, Liang Liu, Ling Tang, Elaine Lai-Han Leung, Pei-Yu Yan. Metabolomics and integrated network pharmacology analysis reveal Tricin as the active anti-cancer component of Weijing decoction by suppression of PRKCA and sphingolipid signaling. Pharmacological Research. 2021,91(71): 105574.DOI：10.1016/j.phrs.2021.105574.
41. Zhang Y G，Zhang A H，Zhang Y，et al. Application of ultra-per-formance liquid chromatography with time-of-flight mass spectrometry for the rapid analysis of constituents and metabolites from the extracts of Acanthopanax senticosus Harms leaf [J]. Pharmacogn Mag，2015，12(46) : 145.
42. A. Asai, M. Konno, J. Koseki, M. Taniguchi, A. Vecchione, H. Ishii, One-carbon metabolism for cancer diagnostic and therapeutic approaches, Cancer Lett. 470 (2020) 141–148.
43. N. Karu, L. Deng, M. Slae, A.C. Guo, T. Sajed, H. Huynh, E. Wine, D.S. Wishart, A review on human fecal metabolomics: methods, applications and the human fecal metabolome database, Anal. Chim. Acta 1030 (2018) 1–24, https://doi.org/10. 1016/j.aca.2018.05.031.
44. Zong-Fang Li, Juan Wang,Chen Huang,Shu Zhang,Jun Yang,An Jiang, Rui Zhou, and Dun Pan. Gas chromatography/time-of-flflight mass spectrometrybased metabonomics of hepatocarcinoma in rats with lung metastasis: elucidation of the metabolic characteristics of hepatocarcinoma at formation and metastasis.Rapid Commun. Mass Spectrom. 2010; 24: 2765–2775.DOI: 10.1002/rcm.4703
45.Yamauchi K, Sakurai H, Kimura T, Wiriyasermkul P, Nagamori S, Kanai Y, Kohno N. System L amino acid transporter inhibitor enhances anti-tumor activity of cisplatin in a head and neck squamous cell carcinoma cell lineCancer Lett. 2009; 276(1):95-101. doi: 10.1016/j.canlet.2008.10.035.
46. Chiang, K.C., Lai, C.Y., Chiou, H.L., Lin, C.L., Chen, Y.S., Kao, S.H., Hsieh, Y.H., 2019. Timosaponin AIII inhibits metastasis of renal carcinoma cells through suppressing cathepsin C expression by AKT/miR-129-5p axis. J. Cell. Physiol. 234 (8), 13332–13341. https://10.1002/jcp.28010.
47.Guowei, Shan Liu, Xinting Zheng, Zhiwei Xiao, Hanrui Chen, Lingling Sun, Chi Zhang, Zhijie Wang, Lizhu Lin. Network Pharmacology/Metabolomics-Based Validation of AMPK and PI3K/AKT Signaling Pathway as a Central Role of Shengqi Fuzheng Injection Regulation of Mitochondrial Dysfunction in Cancer-Related Fatigue. Oxid Med Cell Longev 2021;2021.DOI：10.1155/2021/5556212
48.Ying Zhou, Yaoting Li, Shihao Xu , Jing Lu, Ziyi Zhu , Shaoli Chen, Yuan Tan, Peng He, Jin Xu, Christopher G. Proud, Jianling Xie and Kaikai Shen. Eukaryotic elongation factor 2 kinase promotes angiogenesis in hepatocellular carcinoma via PI3K/Akt and STAT3[J].International Journal of Cancer. 00, 00–00 (2019), DOI: 10.1002/ijc.32560.
49. Chen L, Xiang Y, Kong L, Zhang X, Sun B, Wei X, et al. Hydroxysafflflor yellow A protects against cerebral ischemia-reperfusion injury by anti-apoptotic effect through PI3K/Akt/GSK3b pathway in rat. Neurochem Res 2013;38 (11):2268–75.
50. Guo C，Li S，Liang A，Cui M，Lou Y，Wang H. PPA1 Promotes Breast Cancer Proliferation and Metastasis Through PI3K/AKT/GSK3-beita Signaling Pathway. Front Cell Dev Biol 2021;9. DOI：10.3389/fcell.2021.730558
51. Niimi K, Kohara M, Sedoh E, Fukumoto M, Shibata S, Sawano T, Tashiro F, Miyazaki S, Kubota Y, Miyazaki JI, Inagaki S, Furuyama T. FOXO1 regulates developmental lymphangiogenesis by upregulating CXCR4 in the mouse-tail dermis. Development. 2020; 147:dev181545. https://doi.org/10.1242/dev.181545 PMID:31852686
52. Yadav RK, Chauhan AS, Zhuang L, Gan B. FoxO transcription factors in cancer metabolism. Semin Cancer Biol. 2018; 50:65–76. https://doi.org/10.1016/j.semcancer.2018.01.004 PMID:29309929
53.Pugazhenthi, S.; Nesterova, A.; Sable, C.; Heidenreich, K.A.; Boxer, L.M.; Heasley, L.E.; Reusch, J.E.-B. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J. Biol. Chem. 2000, 275, 10761–10766. [CrossRef]
54. Liu, R.; Chen, Y.; Liu, G.; Li, C.; Song, Y.; Cao, Z.; Li, W.; Hu, J.; Lu, C.; Liu, Y. PI3K/AKT pathway as a key link modulates the multidrug resistance of cancers. Cell Death Dis. 2020, 11, 1–12. [CrossRef]
55. Ortega, M.A.; Fraile-Martí nez, O.; Asú nsolo, Á .; Bujá n, J.; Garcí a-Honduvilla, N.; Coca, S. Signal Transduction Pathways in Breast Cancer: The Important Role of PI3K/Akt/mTOR. J. Oncol. 2020, 2020, 1–11.
56. P.S. Mundi, J. Sachdev, C. McCourt, K. Kalinsky, AKT in cancer: new molecular insights and advances in drug development, Br. J. Clin. Pharmacol. 82 (2016) 943–956.
57. W. Yao, Z. Lin, P. Shi, B. Chen, G. Wang, J. Huang, Y. Sui, Q. Liu, S. Li, X. Lin, H. Yao, Delicaflavone induces ROS-mediated apoptosis and inhibits PI3K/AKT/mTOR and Ras/MEK/Erk signaling pathways in colorectal cancer cells. Biochem. Pharmacol. (Amsterdam, Neth.) 171 (2020) 113680.

Figures:

Figure 1: SNKAF decotion inhibited tumor growth in HMCC-97H cell tumor xenograft models. (A) The anticancer-like activity of SNKAF decotion was evaluated in nude mice with xenografted tumors. A total of 30 mice were subcutaneously injected with HMCC-97H cells, and then randomly assigned to five groups: (1) model group (treated with saline), three SNKAF decotion dosage and Sorafenib groups. (B) The mice were sacrificed for tumor tissue collection and the tumors were weighted after 15 days of administration. (C) The tumor volume was measured every two days. (D) The mice weight was measured every 2 days before expression and the weight was measured every other day when drug treatment. Error bars represent the mean ± SE. (E) The H&E staining, (F) IHC detection of Ki67 expressions from the indicated groups., ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *v.s.* model).

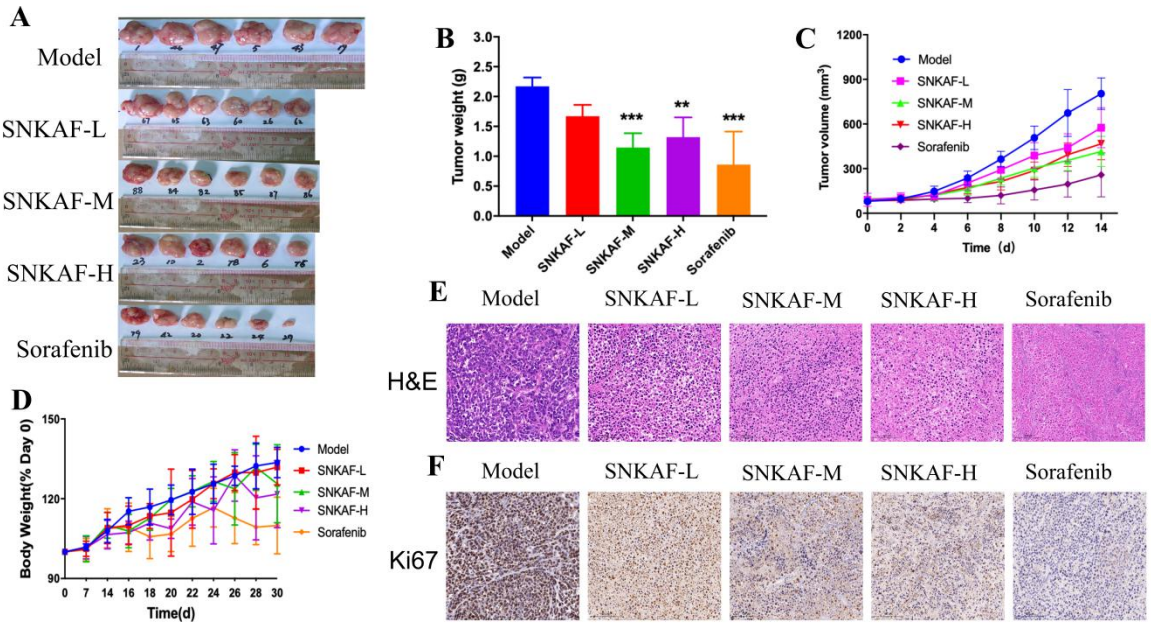


Figure 2: SNKAF serum inhibited the proliferation, migrative and invasive potentia of HCC cells. (A,B) MHCC97H and HCCLM3 cells were treated with a gradient concentration of SNKAF serum, rat blank serum and sorafenib for 48 h or certain time intervals (0, 24, 48 and 72 h) by CCK8 assays. (C) The influence of SNKAF serum on colony formation of MHCC97H and HCCLM3 cells. (D) phase-contrast images for wound healing after administration SNKAF serum for 48 h. right: quantification of migration healing areas.(E) images (left) and quantification (right) of decreased cell number in transwell chambers with or without SNKAF serum on MHCC97H and HCCLM3 cells.

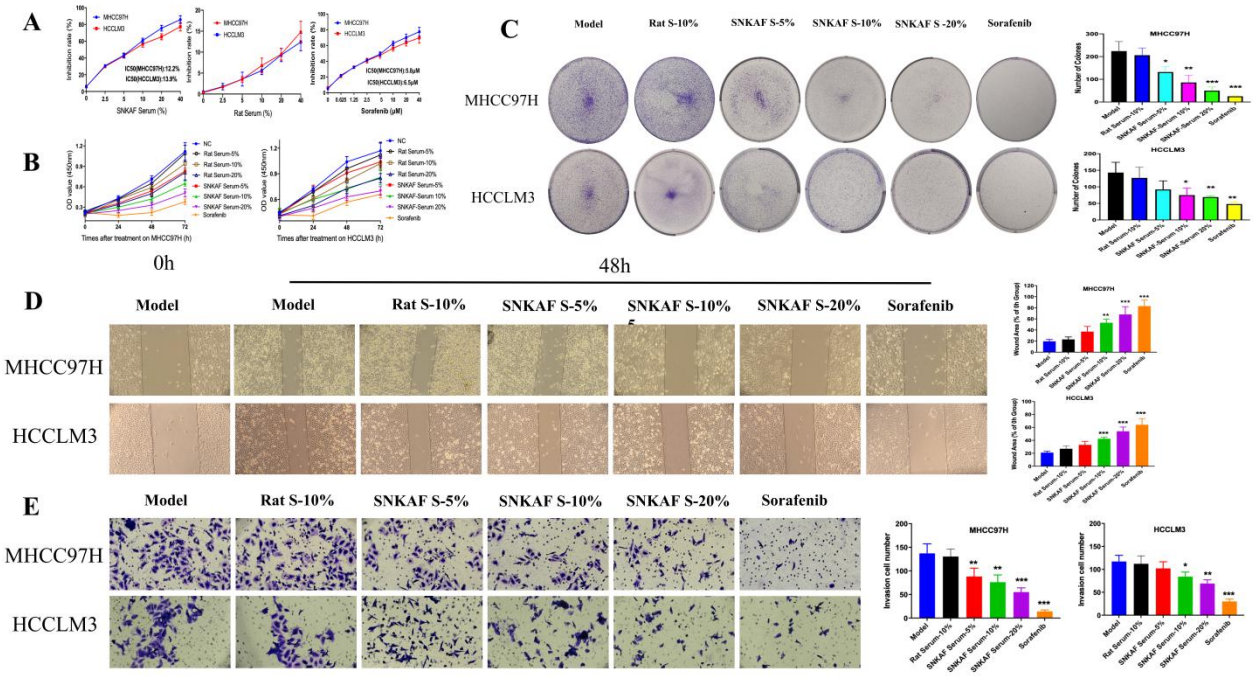


Figure 3: SNKAF Serum Induced Apoptosis in HCC Cells and Arrest HCC Cell Cycle at the S and G2/M Checkpoint. Cell cycle demonstrated different dosage of SNKAF serum relieved the G2/M and S arresting in both MHCC97H and HCCLM3 cells using flow cytometry(A). Apoptosis analysis of (B) the MHCC97H and HCCLM3 cells treated with SNKAF serum, rat serum and sorafenib using flow cytometry. Hoechst 33258 staining(C) with TUNEL assay (D) showed typical apoptotic morphology changes of cells after indicated treatment.

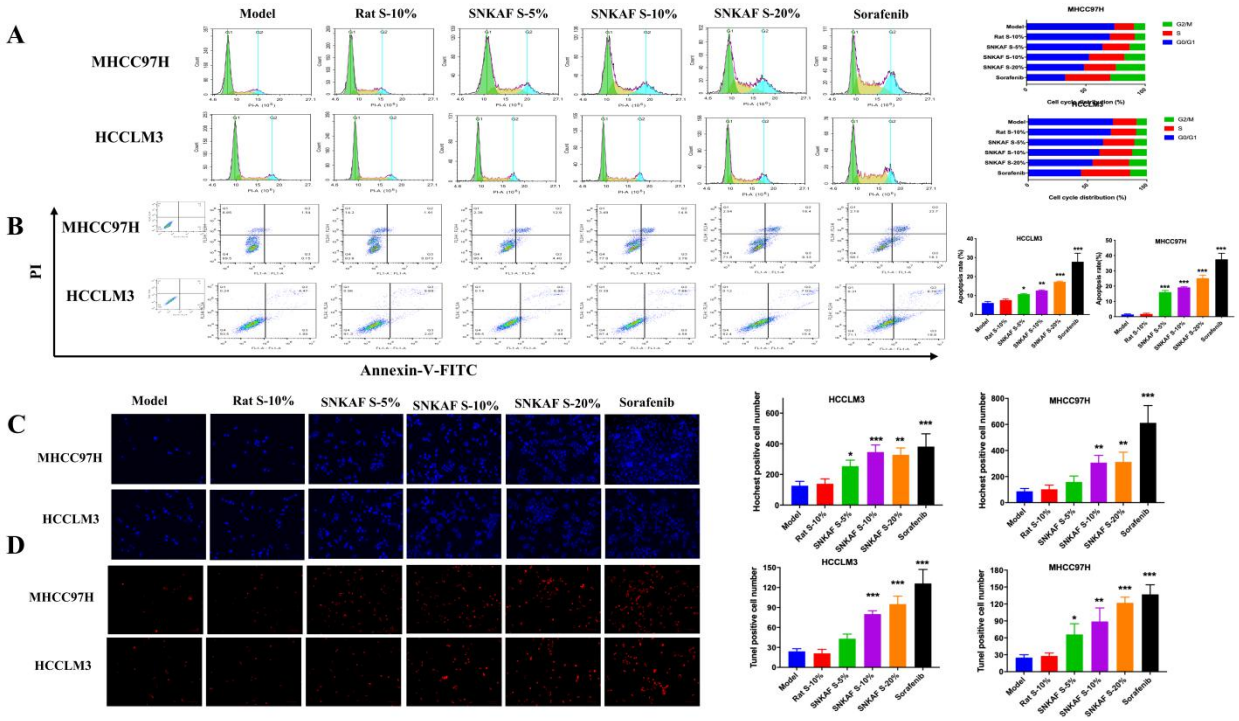


Figure 4:Network pharmacology analysis of SNKAF treating HCC. (A) The venn analysis showed the crosstalk of DEGs in SNKAF and hepatocellular carcinoma; (B) PPI network of herb's compound targets against HCC, The nodes with red borders represent the hub genes. (C) The GO enrichment analysis of potential targets by ClueGO, all data collected have a p-value ≤0.01. (D) The KEGG pathways enrichment analysis by ClueGO. All pathways have a p-value of ≤0.01. (E) Gene target-pathway signal network, the green ellipse represents the different gene targets and the square colors represent the pathways.

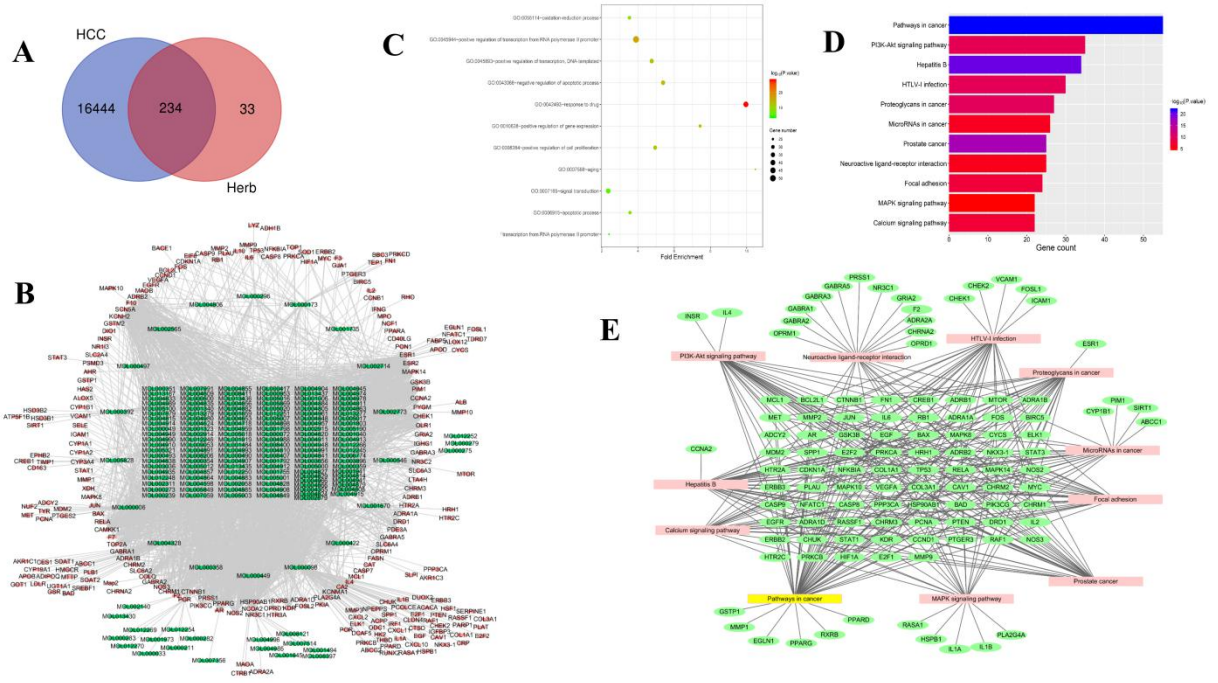


Figure 5: | BPI chromatograms of the SNKAF Serum groups and control groups in the positive positive —and negative —ion modes.

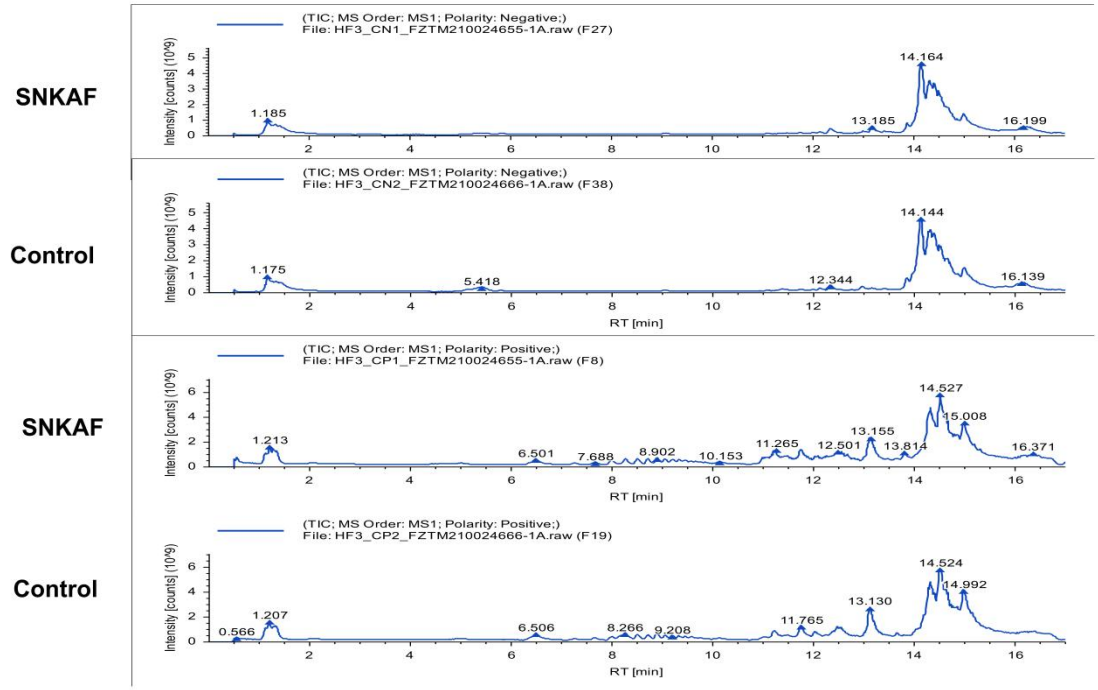


Figure 6: Differential metabolites in plasma were identified between the SNKAF group and the control group. （A) 3D-PCA plot based on data from the Control and SNKAF groups obtained in the positive (right)—and negative (left)—ion modes. (B) PLSDA plot based on data from the Control, SNKAF groups obtained in the positive positive (right)—and negative (left)—ion modes. (C) Volcano-plots of metabolites data from the Control, SNKAF groups obtained in the positive positive (right)—and negative (left)—ion modes; (D) VIP plots of fecal samples from the Control, SNKAF groups obtained in the positive positive (right)—and negative (left)—ion modes; (E) Heatmap of difference metabolites data from the Control, SNKAF groups obtained in the positive positive (right)—and negative (left)—ion modes.

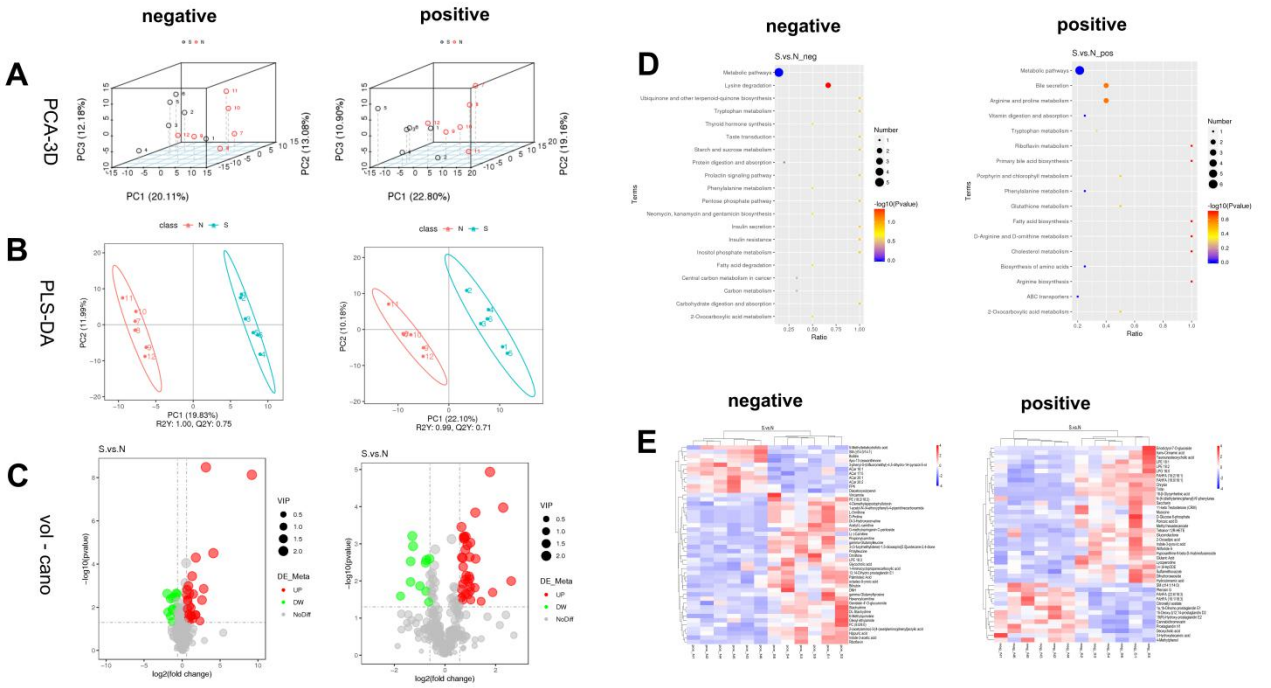


Figure 7:Combined network pharmacology and metabolomics to analyze the target of SNKAF on hepatocellular carcinoma. (A) The venn analysis showed the crosstalk of DEGs in two omics, (A: metabolomics, B: network pharmacology); (B) PPI network of HCC targets and (C) Top five clustering graphs from the PPI network of HCC targets.(D)The GO enrichment analysis of potential targets by ClueGO; (E) Top 20 significantly enriched pathways enrichment by KEGG analysis; (F) KEGG pathway suggested that various targets in PI3K/AKT signaling were tightly associated with SNKAF pharmacological action. The red rectangle nodes represent the most significant genes or biological pathways associated with SNKAF pharmaoclogical action.

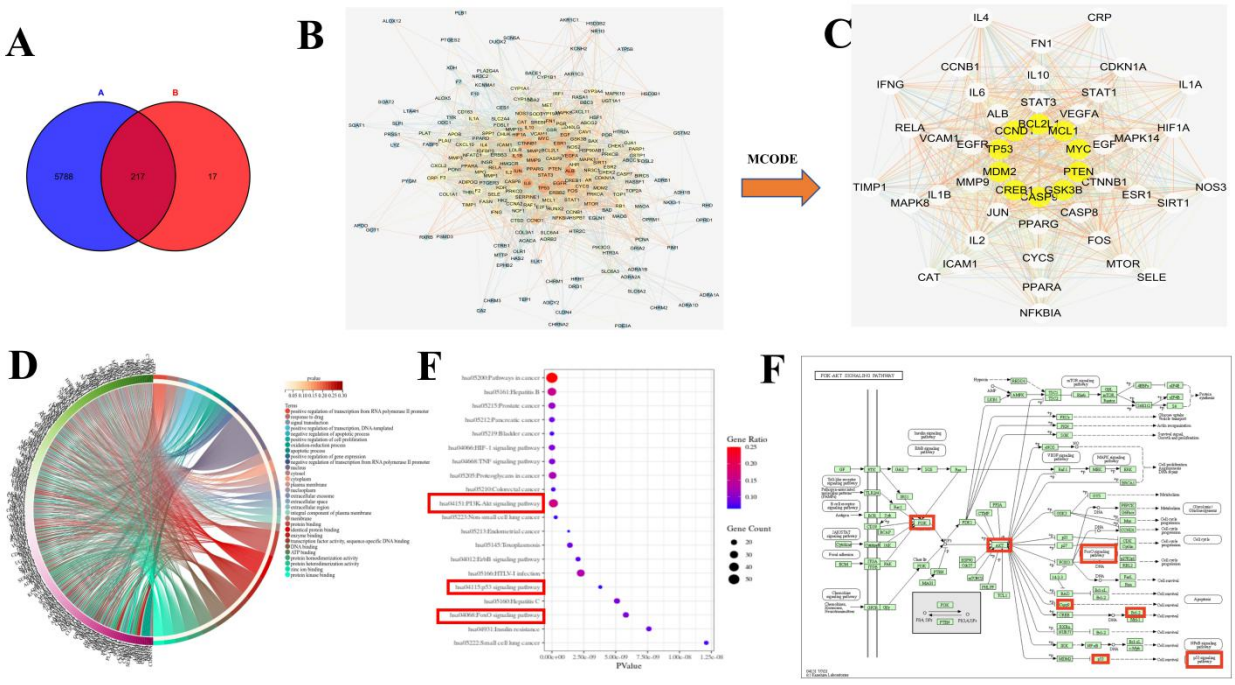
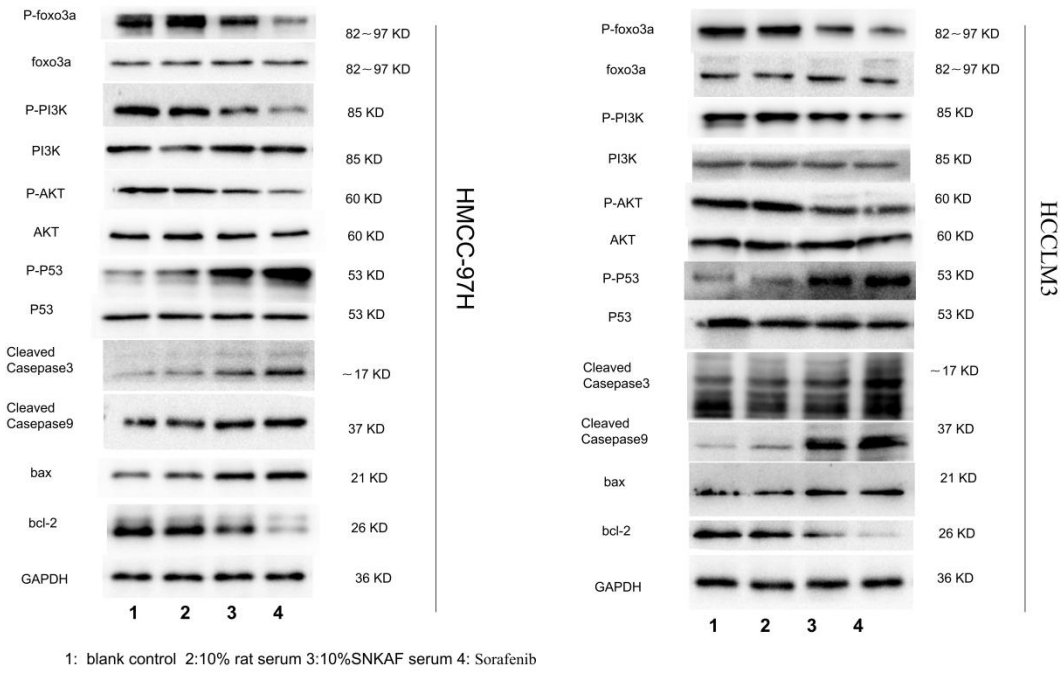
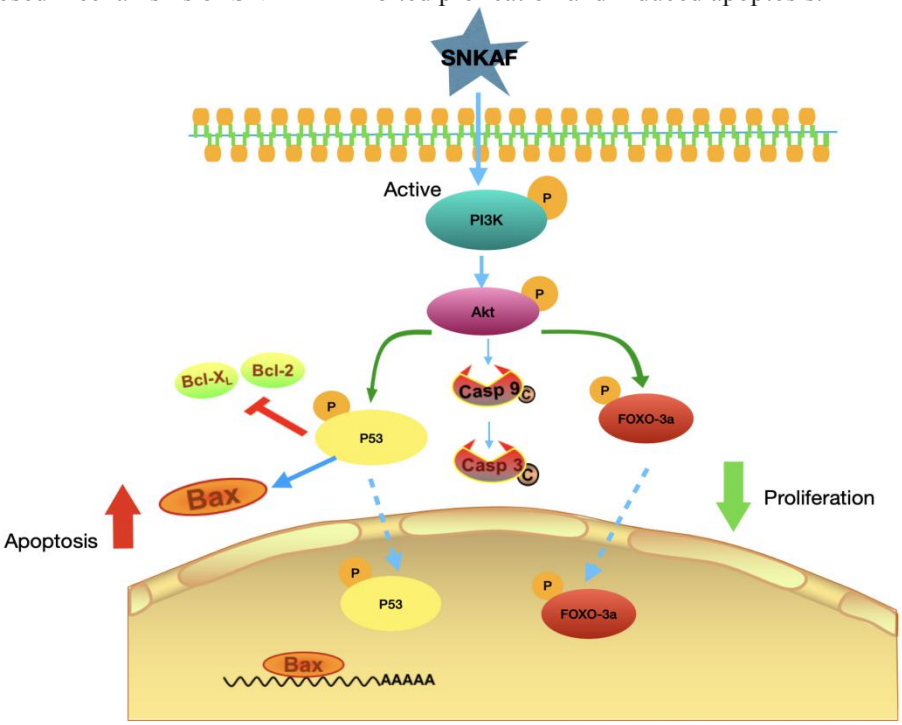


Figure 8: Validation and Identification of SNKAF as a Anti-cancer Inhibitor in Suppressing HCC Apoptosis and Proliferation Through the PI3K/AKT/P53/FoxO Axis. Protein expression by Western blot assay. The expression of P-Fox3a/Fox3a, p-PI3K/PI3K, p-Akt/Akt, P-p53/p53, Bax, Bcl-2,cleaved-caspase-9 and cleaved-caspase-3 using western blotting analysis after indicated treatment in HCCLM3 and MHCC97H cells; Lanes 1–4: blank control; 10% rat serum; 10% SNKAF serum; sorafenib.



1: blank control 2:10% rat serum 3:10%SNKAF serum 4: Sorafenib

Figure 9: The proposed mechanisms of SNKAF-inhibited prolication and induced apoptosis.



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementTable1thepotentialactivecompounds.docx
- SupplementaryTable3.et