

Phillyrin regulates the JAK2/STAT3 signaling pathway by inhibiting TOP2A expression to accelerate ferroptosis in hepatocellular carcinoma

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Abstract. Despite advancements and refinements in the therapeutic approaches for hepatic malignancies, liver cancer remains a prevalent and deadly form of cancer, with its grim outlook posing as a significant clinical challenge. Phillyrin (PHN) has been reported to have anticancer effects, but the anticancer mechanism in liver cancer is ominous. By searching the potential target of PHN in the online database and liver cancer disease database, it was found that there is only one overlap gene, and DNA topoisomerase II alpha (TOP2A) is abnormally expressed in liver cancer tissues. TOP2A overexpression and downregulated hepatocellular carcinoma cell lines were then constructed *in vitro*, and it was examined whether PHN treatment induced ferroptosis in hepatocellular carcinoma by regulating TOP2A's inhibition of Janus kinase 2/Signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway through phenotypic assay, western blot assay, reverse transcription-quantitative PCR assay and electron microscopy. The results showed that PHN could inhibit the expression of TOP2A protein and JAK2/STAT3 signaling pathway in hepatoma cells. PHN could also downregulate glutathione peroxidase 4 by suppressing the expression of TOP2A protein. PHN impeded the activity of factor inhibiting hypoxia-inducible factor 1 alpha, thereby augmenting the synthesis of iron-dependent apoptosis-related

proteins including cytochrome c oxidase subunit II, long-chain acyl-CoA synthetase family member 4 and NADPH oxidase 1, thus facilitating an increase in Fe²⁺ concentration and accelerating oxidative harm within hepatocellular carcinoma cells, culminating in the induction of ferroptotic cell death in these liver malignancy cells.

Introduction

Hepatic carcinoma represents a prevalent form of cancer, with both its occurrence and fatality rates experiencing a steady climb globally (1). Projections indicate a 55.0% surge in the annual incidence of new liver cancer cases from 2020 to 2040, with an anticipated 1.4 million diagnoses in 2040. Additionally, it is estimated that by 2040, 1.3 million individuals will succumb to liver cancer, marking a 56.4% increase from the figures in 2020 (2). Among the various types of liver cancer, hepatocellular carcinoma (HCC) dominates, comprising roughly 90% of all liver cancer cases (3). The causes of HCC are complex, including hepatitis virus infection, non-alcoholic steatohepatitis and cirrhosis (4). HCC is often asymptomatic in its early stages, which leaves numerous patients undetected and untreated. Once symptoms appear, such as hepatic pain, jaundice and ascites, HCC is often advanced, difficult to treat, and has a poor prognosis (5). There is an urgent need to find new targeted drugs to treat and prevent HCC.

Natural medicine is a treasure house to create targeted medications with minimal toxicity and superior efficacy. *Forsythia suspensa* extract, known as phillyrin (PHN), is derived from the desiccated berries of the Forsythia plant, belonging to the Oleaceae family (6). Over the past few years, there has been substantial advancement in the investigation of its therapeutic properties, including its anti-inflammatory and antioxidant capabilities, and cardiovascular protection properties (7-9). PHN can protect against alcoholic steatohepatitis injury and activate the liver kinase B1/AMP-activated protein kinase (AMPK) signaling pathway in HepG2 cells to reduce lipid accumulation induced by high glucose in human hepatocytes (10,11). In addition, PHN also has certain anticancer

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effects. PHN can inhibit the ferritin heavy chain 1/solute carrier family 7 member 11 axis to sensitize lung cancer cells to ferroptosis (12), and PHN combined with autophagy blockers can alleviate laryngeal squamous cell carcinoma through AMPK/mTOR/p70S6K signal transduction (13). In general, PHN has shown some potential in the treatment of liver diseases and has anticancer activity, but the role of PHN in HCC remains unclear.

The research field of ferroptosis, a mode of cell death dependent on iron ions, has grown exponentially in the past few years, and its mechanisms mainly include iron-dependent reactive oxygen species (ROS) production and iron-dependent protein degradation (14). The occurrence of ferroptosis in HCC may be related to factors such as iron homeostasis imbalance, oxidative stress and elevated intracellular iron ion concentration (15). In addition, changes in some molecular targets, such as ferric regulatory proteins and antioxidant enzymes, may also participate in the occurrence of ferroptosis (16). It has been reported that PHN can activate the sensitivity of lung cancer cells to ferroptosis and inhibit cell proliferation (12), but no studies have pointed out the regulatory relationship between PHN and ferroptosis of liver cancer cells. Over the past few years, an increasing body of research has highlighted the pivotal role of the JAK2/STAT3 signaling cascade in the development of HCC (17). Within hepatoma cells, the stimulation of the JAK2/STAT3 signaling axis fosters cellular multiplication and curbs programmed cell death, thereby facilitating the growth of malignant tumors (18). The erratic activation of the JAK2/STAT3 pathway within HCC represents a crucial hurdle in the therapeutic management of this disease (19). Extensive investigations have demonstrated that *Forsythia suspensa* extract possesses therapeutic properties against diverse maladies through its regulation of the JAK2/STAT3 pathway (20–22). Consequently, the present investigation was centered on elucidating the precise function of PHN in HCC, as well as exploring the initial mechanisms behind the iron-induced apoptosis in hepatocytes and its connection to the JAK2/STAT3 signaling pathway.

Materials and methods

GSE136247 data set differential gene volcano map and heat map. The Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/gds>) was used to download GSE136247 dataset (23), using R package ‘FactoMineR’ (<https://cran.r-project.org/web/packages/FactoMineR/index.html>), ‘factoextra’ (<https://cran.r-project.org/web/packages/factoextra/index.html>). An advanced multivariate statistical method, known as Principal Component Analysis (PCA), was utilized to analyze the data compilations derived from the two disparate groups of sample cohorts. Subsequently, the ‘limma’ (<https://www.bioconductor.org/packages/release/bioc/html/limma.html>) package was utilized to delve into the disparities in gene expression between these two cohorts. A total of 527 differentially expressed genes were obtained according to $P < 0.05$ and $|\log FCI| > 1$. A graphical representation of the gene volcano plot was crafted utilizing the R library ‘ggplot2’, whereas the graphical depiction of the Top 20 differentially expressed genes volcano plot was created with the assistance of the R package ‘pheatmap’.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) path enrichment analysis of GSE136247 dataset. For 527 genes from the GSE136247 dataset, GO and KEGG functional enrichment was performed using the R package ‘clusterProfiler’ (<https://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html>), and the top-ranking results were displayed.

Protein-protein interaction networks (PPI) network diagram of the GSE136247 dataset. The STRING repository (accessed at <https://cn.string-db.org/>) was employed to build a network of protein interactions utilizing the aforementioned 527 overlapping genes.

Expression and prognosis of (DNA topoisomerase II alpha) TOP2A in the cancer genome atlas (TCGA) liver cancer cohort. The GEPIA2 (<http://gepia2.cancer-pku.cn/#analysis>) database was accessed, which provides cancer gene expression data based on the TCGA database. The name of the TOP2A gene was input and HCC [TCGA-liver hepatocellular carcinoma (LIHC)] was selected as the research object. In the expression analysis section, GEPIA2 was used to draw the expression comparison chart of TOP2A in liver cancer tissues and normal liver tissues, and box plots were used to demonstrate the expression differences between different groups. In order to assess the impact of TOP2A expression on the survival outcomes of liver cancer sufferers, the GEPIA2 database survival analysis feature was utilized. Patients with liver cancer were categorized into two groups based on the level of TOP2A expression: One with elevated expression and the other with reduced expression. Through the online website Kaplan-Meier Plotter (<https://kmplot.com/analysis/>), ‘Liver cancer’ was selected, the gene name ‘TOP2A’ was input, the follow-up time endpoint was set to sixty months in ‘Follow up threshold’, and the survival curve was drawn. The log-rank method was employed to compare the survival discrepancies between these two subsets, aiming to establish a link between TOP2A expression levels and the patients’ prognostic outlook.

PHN is docked with TOP2A molecules. Initially, the three-dimensional structure of TOP2A (with PDB identifier: 1ZXN) was sourced from the Protein Data Bank (PDB; <https://www.rcsb.org/>). The PHN file in SDF format was retrieved from the PubChem database (accessible at <https://pubchem.ncbi.nlm.nih.gov>). Subsequently, Open Babel (<http://openbabel.org/index.html>) was utilized to transform this file into the PDB format. Following this, the protein underwent dehydration and hydrogenation processes, after which its charge was computed. Using Autodock tools version 1.5.7 (<https://autodock.scripps.edu/adt/>), the protein was then formatted into pdbqt.

The 3D structural data of TOP2A, identified by PDB code 1ZXN, was acquired from the PDB repository. The Small Molecule Data File (SDF) for PHN was fetched from the PubChem database, and then converted into PDB format via Open Babel. The protein structure was subsequently stripped of water molecules and hydrogenated, with its electrical charge assessed. The conversion to pdbqt format was facilitated by Autodock tools version 1.5.7. Ligand processing involved hydrogenation and the calculation of torsional energies,

culminating in its transformation into pdbqt format as well. The boundaries for the docking simulation were set, and the molecular docking simulation was carried out using Autodock vina. Visual inspection and three-dimensional analysis were conducted with pymol 2.1.0 (Schrödinger Corporation), yielding a comprehensive 3D graphical representation.

Cell culture. Human HCC cell lines JHH7, HEP3B217, SNU878, JHH2 and normal liver cell line THLE-2 were purchased from the Qingqi (Shanghai) Biotechnology Development Co., Ltd. The frozen cells were thawed after retrieval from liquid nitrogen storage and revitalized. These cells were subsequently cultivated in a DMEM solution (cat. no. 11995; Beijing Solarbio Science & Technology Co., Ltd.), which consisted of a blend of 10% newborn calf serum (cat. no. S9020; Beijing Solarbio Science & Technology Co., Ltd.) and 1% penicillin-streptomycin mixture. PHN (cat. no. F798884) was purchased from Shanghai Maclin Biochemical Technology Co., LTD.

Cellular transfection. The T25 cell culture vial was removed from the cell incubator and the vial contained the exponential growth stage of the liver cancer cell line. The old medium was discarded, 2 ml sterile PBS was added, the T25 cell culture bottle was gently shaken, the excess PBS was discarded, and then 1 ml trypsin digestion solution (cat. no. T1300; Beijing Solarbio Science & Technology Co., Ltd.) was added and incubated at 37°C for 2 min. Digestion was terminated by adding 2 ml DMEM medium. The cells were counted by Thermo Fisher Scientific, Inc. (Countess 3) and inoculated into six wells with 1×10^5 cells per well and placed in a cell incubator at 37°C (Countess 3; Thermo Fisher Scientific, Inc.) overnight. The old medium was discarded, add the premixed solution of Lip2000 (cat. no. 11668030; Thermo Fisher Scientific, Inc.) and SiTOP2A interference sequence (1,500 ng per well) or overexpression plasmid (2,500 ng per well) were added and cells were cultured in serum-deprived DMEM solution for a period of 5 h. Subsequently, fresh DMEM was introduced, and the culture was maintained for an additional 48 h to proceed with the subsequent investigation. The specific sequences for the small interfering RNA (siRNA) and plasmid are presented in Table SI.

Group information for cell experiments. siRNA was used to knockdown TOP2A (si-TOP2A), and plasmids were used for overexpression of TOP2A (OE-TOP2A). In JHH7 cells, they were divided into three groups: Si-negative control (NC), PHN + Si-NC, and PHN + si-TOP2A. In the PHN + si-TOP2A group, JHH7 was transfected with siRNA and incubated for 48 h. The Si-NC and PHN + Si-NC groups were transfected with the control sequence and incubated for 48 h. After that, PHN was added to the PHN + Si-NC and PHN + si-TOP2A groups and incubated for another 48 h. Cells were extracted for subsequent experiments. In JHH2 cells, they were divided into three groups: Vector, Vector + PHN, and OE-TOP2A + PHN. In the Vector + PHN and OE-TOP2A + PHN groups, PHN was added to JHH2 cells and incubated for 48 h. After that, the OE-TOP2A + PHN group was transfected with the overexpression TOP2A plasmid and continued to be incubated for 48 h. The Vector and Vector + PHN groups were transfected with the control vector and continued to be incubated for 48 h. Cells were extracted for subsequent experiments.

Cell counting kit-8 (CCK-8) assay. Cell transfection is performed in the same way as aforementioned. The successfully transfected cells were removed from the incubator, cell digestion and centrifugation were consistent with previous methods, and cell precipitation was collected. The cultures were seeded into 96-microwell trays at a seeding density of 3,000 cells per well and subsequently exposed to varying dosages of PHN for periods of 0, 24, 48 and 72 h. Following this treatment, 10 μ l of the CCK-8 solution (cat. no. C0038; Beyotime Institute of Biotechnology) was introduced into each individual well, after which the incubation at 37°C was prolonged for an additional 4 h. Finally, the 96-well plates were removed from the cell incubator and placed on the SpectraMax Mini multi-function enzyme label to detect OD (450 nm) value (Molecular Devices, LLC). The subsequent data were statistically analyzed by Graph Prism 9.5.0 software (Dotmatics).

EDU staining. Exponential growth cells were received, cell digestion was carried out, add 1×10^5 cells per well into six-well plates and incubated overnight in a cell incubator. Transfection EDU solution (10 μ mol/l; cat. no. C0071S; Beyotime Institute of Biotechnology) was added to the transfected cells and incubated in a cell incubator for 2 h. The old medium was removed, and 1 ml of cell fixing solution (cat. no. P0099; Beyotime Institute of Biotechnology) was added to each well for incubation at room temperature (20°C) for 30 min, and then 1 ml of 0.3% Triton X-100 (cat. no. P0096; Beyotime Institute of Biotechnology) was added to each well and incubated at room temperature (20°C) for 10 min. The permeable solution was removed and 1 ml Hoechst 33342 solution (cat. no. C1022; Beyotime Institute of Biotechnology) was added to each well and incubated for 10 min at room temperature (20°C) without light. The Hoechst 33342 solution was removed, cleaned with PBS for 3 times, observed with inverted fluorescence microscope (model AMF7000; Thermo Fisher Scientific, Inc.). Images were captured, and then statistical analysis was performed using Image J 1.52a (National Institutes of Health).

Reverse transcription-quantitative (RT-qPCR) assay. Transfection of HCC was performed by adding a suitable volume of TRIzol reagent (cat. no. 15596026; Beijing Solarbio Science & Technology Co., Ltd.) was utilized for the isolation of total RNA, adhering to the provided protocol. Subsequently, the RNA concentration was assessed using the NanoDrop One spectrophotometer. Following this, complementary DNA was synthesized through reverse transcription, following the manufacturer's protocol (cat. no. D7168M; Beyotime Institute of Biotechnology). Thereafter, the BeyoFast™ SYBR Green RT-qPCR Mix (cat. no. D7262; Beyotime Institute of Biotechnology) was incorporated into the reaction mixture. The template DNA was fully denatured by pre-heating at 95°C for 3 min on the PCR apparatus, and then entered the amplification cycle. In each cycle, the template was denatured by holding at 95°C for 30 sec, and then the temperature was lowered to 60°C for 30 sec, so that the primer and the template were fully annealed. The mixture was held at 72°C for 1 min (primer extended on the template, DNA synthesized, a cycle complete, then repeat 40 cycles) and finally set 4°C. The 7900HT fluorescence quantitative PCR instrument

(cat. no. 4351405; Thermo Fisher Scientific, Inc.) was set up according to the instructions. Relative gene expression data were analyzed by real-time quantitative PCR and the $2^{-\Delta\Delta C_q}$ method (24). The subsequent data were statistically analyzed by Graph Prism 9.5.0 software. Details of the primer sequences are described in Table SI.

Fe²⁺ content determination and malondialdehyde (MDA) detection. HCC cell transfection was conducted as aforementioned; cells in each group were expanded to $>2 \times 10^6$ to extract cell precipitation for use. The HCC cell specimens underwent processing in compliance with the iron concentration testing kit (cat. no. MAK025; MilliporeSigma). The intracellular Fe²⁺ concentration was assayed, and the optical density (OD) at 593 nm was obtained using the SpectraMax Mini multi-purpose enzyme analyzer. Additionally, the cells affected by HCC were subjected to the MDA measurement protocol as per the kit instructions (cat. no. S0131S; Beyotime Institute of Biotechnology). The OD readings at 532 nm were recorded with the aid of the SpectraMax Mini multifunctional enzyme analyzer. The subsequent data were statistically analyzed by Graph Prism 9.5.0 software.

Transmission electron microscopy (TEM). After treating each group of cells by the aforementioned method, the cells were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide for 2 h respectively at 4°C in sequence, and then dehydrated in a series of acetone gradients. Subsequently, the morphological changes of mitochondria in the tissues were observed by transmission electron microscopy (JEM-1400FLASH; JEOL, Ltd.), and the images were captured.

ROS staining. The successfully transfected JHH7 and JHH2 cells were inoculated in six-well plates at 1×10^5 cells/well, and incubated overnight. Then, the culture medium was removed and the cells were gently washed twice with PBS. Next, 2 ml of ROS fluorescent working solution (cat. no. S0033M; Beyotime Institute of Biotechnology) were added, and the mixture was incubated for 30 min away from light. After the incubation, the cells were washed again with PBS to remove the ROS staining solution. Subsequently, DAPI staining solution (1 µg/ml) was used to stain the cell nuclei, and cells were incubated away from light for 5 min. After the staining was completed, the cells were washed with PBS. Finally, images were observed and captured under a fluorescence microscope, and the relative expression level of intracellular ROS was analyzed using ImageJ 1.5.2a software (National Institutes of Health).

Western blotting (WB). The treated cells were selected, and these cell samples were co-incubated with RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) at 4°C for 30 min. Subsequently, protein extracts were isolated from HCC cells. The protein levels were quantified using a BCA assay kit. Based on the protein concentration readings, the volume of the sample was adjusted correspondingly. An equal volume of the protein (30 µg) mixture was pipetted into the wells of the gel matrix (containing 30% acrylamide), and the electrical potential was fixed at 120 volts for the purpose of conducting gel electrophoresis. Following a 75 min run, the gel was transferred onto a PVDF membrane,

and the electrical setting was then increased to 200 volts. After a 30 min transfer, the PVDF membrane was immersed in a solution of 5% non-fat milk powder and allowed to sit for a duration of 2 h at 25°C. TBST (containing 0.1% Tween 20) was cleaned three times after incubation, TBST was cleaned three times after the primary antibody was incubated overnight at 4°C, and chemiluminescent solution was added for 10 sec after the secondary antibody was incubated for 2 h at 25°C. Images were stored in the chemiluminescence apparatus (SH-Cute523; Hangzhou Shenhua Technology Co., Ltd.) for subsequent statistical analysis. WB antibody information is included in Table SII. Densitometric analysis was carried out using ImageJ 1.5.2a software (National Institutes of Health).

Statistical analysis. All statistical evaluations were conducted with the SPSS version 22.0 program (IBM Corp.). Quantitative results are presented as the average value $\pm$ standard deviation. For comparisons between the two distinct groups, an independent samples t-test was applied. To assess variations among several groups, a one-factor ANOVA followed by Tukey's multiple comparison test was utilized. Comparisons across various time intervals were executed with the Bonferroni correction for post hoc analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GSE136247 differential expression analysis. PCA was first performed on GSE136247 data to obtain a total of 527 differentially expressed genes, of which 93 were upregulated and 434 downregulated (Fig. 1A). Subsequently, differential genes were selected to draw volcano maps (Fig. 1B) and cluster heat maps (Fig. 1C). It was found that compared with normal liver tissues, AFP, PEG10, SULT1C2, GPC3 and acyl-CoA synthetase family member 4 (ACSL4) were most significantly upregulated in liver cancer tissues, while MT1G, MT1G, C9, CYP2C8 and CTP1A2 were most significantly downregulated. These significant differential genes can be used as potential therapeutic targets for liver cancer. KEGG functional enrichment analysis showed that differential genes were mainly enriched in complement and coagulation cascades, drug metabolism and metabolism of xenobiotics by cytochrome P450 (Fig. 1D and E). Subsequently, differential gene PPI network interaction was conducted (Fig. 1F).

Screening of potential therapeutic targets for PHN. The differential genes of liver cancer tissue and normal liver tissue obtained as aforementioned were intersected with PHN drug targets to draw Wayne's diagram. It was found that only one intersection gene was TOP2A (Fig. 2A). Later, the levels of TOP2A expression in TCGA liver cancer dataset (TCGA-LIHC) were investigated and a significant increase was observed in TOP2A levels within liver cancer samples compared with the normal tissue controls (Fig. 2B). This observation aligns with the current analysis of TOP2A expression patterns. Moreover, there was a significant association between TOP2A expression and liver cancer outcomes, with patients exhibiting high TOP2A expression demonstrating poorer prognostic outcomes compared with those with low TOP2A expression levels (Fig. 2C). The 2D and 3D molecular

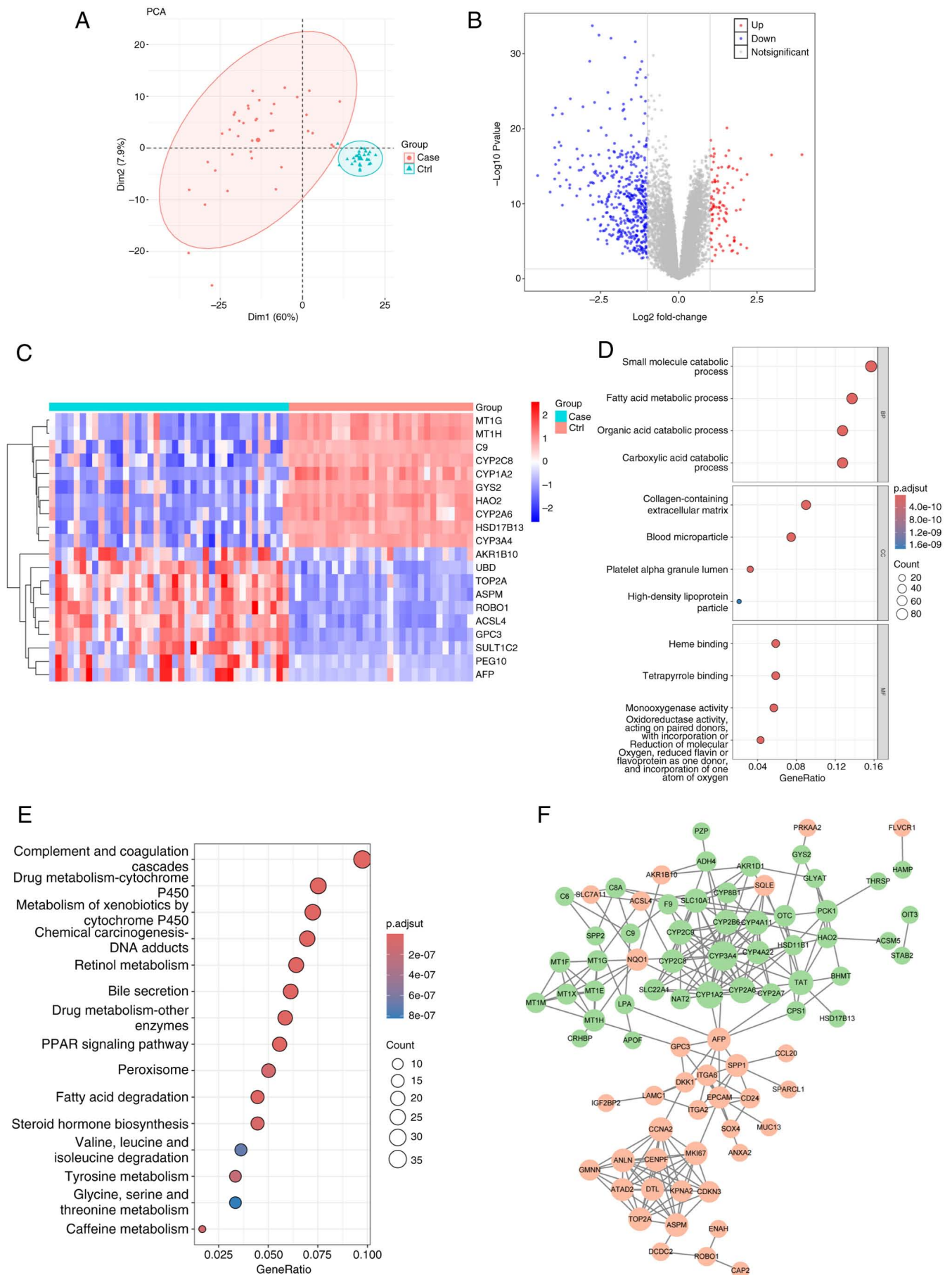


Figure 1. Differential gene analysis of hepatocellular carcinoma in the GSE136247 dataset. (A) GSE136247 principal component analysis of the dataset. (B) Varied gene eruption plot. (C) Conglomerate gene thermal representation. (D) Distinct Gene Ontology categorization enhancement study. (E) Unique gene Kyoto Encyclopedia of Genes and Genomes pathway enhancement examination. (F) Differential gene protein-protein interaction network analysis.

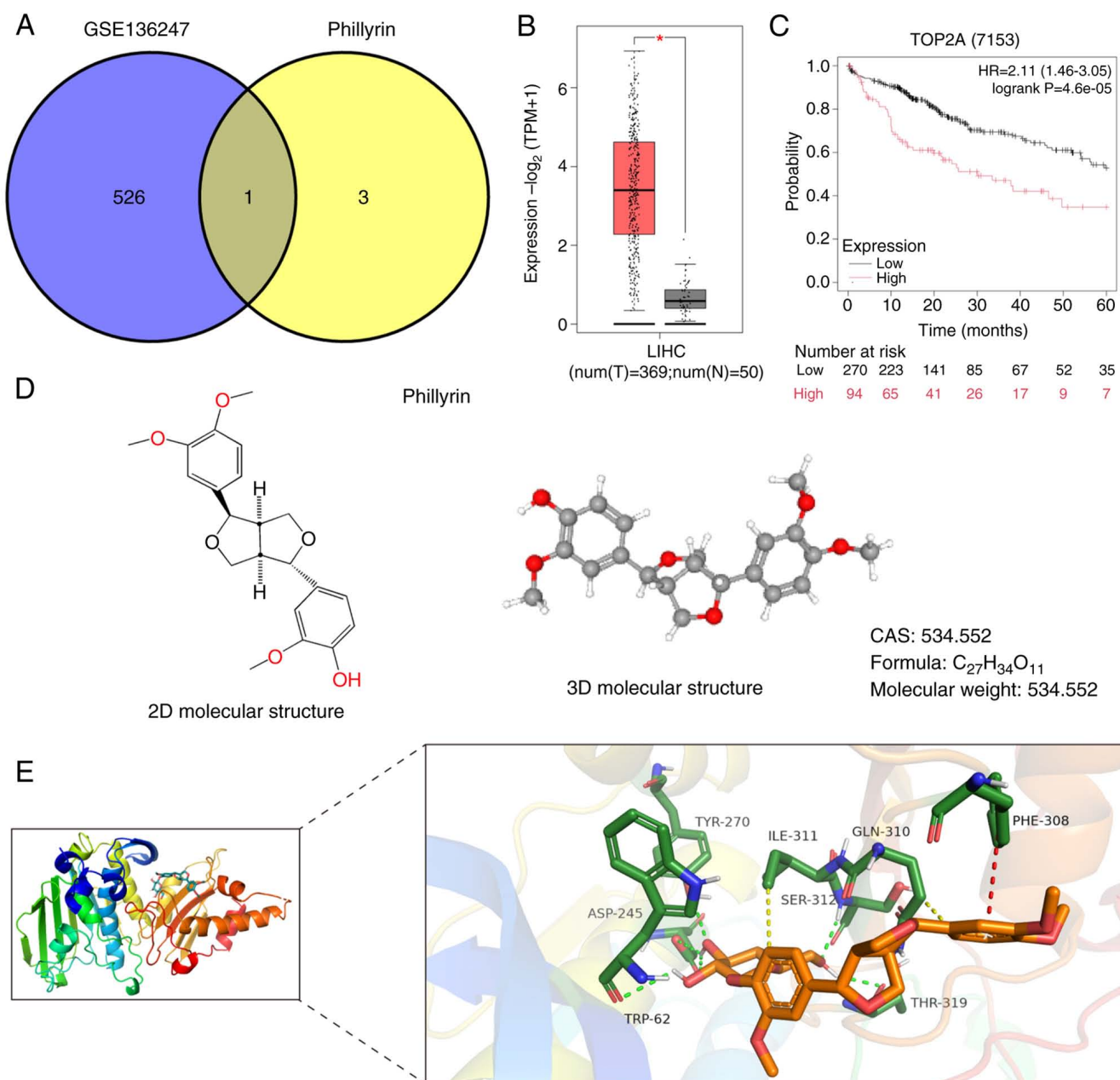


Figure 2. Potential target gene prediction for PHN treatment in hepatocellular carcinoma. (A) GSE136247 differential gene and PHN drug target were used to draw Venn map. (B) Expression of TOP2A in TCGA cohort. (C) Prognostic analysis of TOP2A in TCGA cohort. (D) PHN 2D and 3D molecular structure and relative molecular mass. (E) Schematic diagram of docking PHN with TOP2A protein molecules. * $P < 0.05$. PHN, phillyrin; TOP2A, DNA topoisomerase II α ; TCGA, The Cancer Genome Atlas.

structure formula of PHN is demonstrated in Fig. 2D. In order to further confirm the existence of binding sites between PHN and TOP2A, molecular docking was simulated *in silico*, and the docking results showed that there were binding sites between PHN and TOP2A, along with a strong binding effect (Fig. 2E).

Screening of liver cancer cell lines. Through our online database (<https://sites.broadinstitute.org/ccle/>), the different TOP2A expression was screened in HCC cell line (Fig. 3A). The pair of HCC cell lines exhibiting the most prominent contrast in TOP2A expression levels was chosen, along with healthy liver cells, to investigate TOP2A expression via WB and RT-qPCR analysis. The findings indicated that TOP2A

was most prominently expressed in the JHH7 cellular line while the expression was lowest in JHH2 cells (Fig. 3B). This further proves the accuracy of the database prediction information. Therefore, JHH7 cells with high TOP2A expression and JHH2 with low TOP2A expression were selected for subsequent experiments.

PHN can inhibit the proliferation of TOP2A and liver cancer cells. To verify the effects of PHN on the proliferation of JHH7 and JHH2 HCC cells, the effects of different concentrations (6.25, 12.5, 25, 50, 100 and 200 $\mu\text{mol/l}$) of PHN on HCC cell proliferation were examined after 24 h of treatment by CCK-8 assay. The results of CCK-8 revealed that PHN had no detailed inhibitory effect on the viability

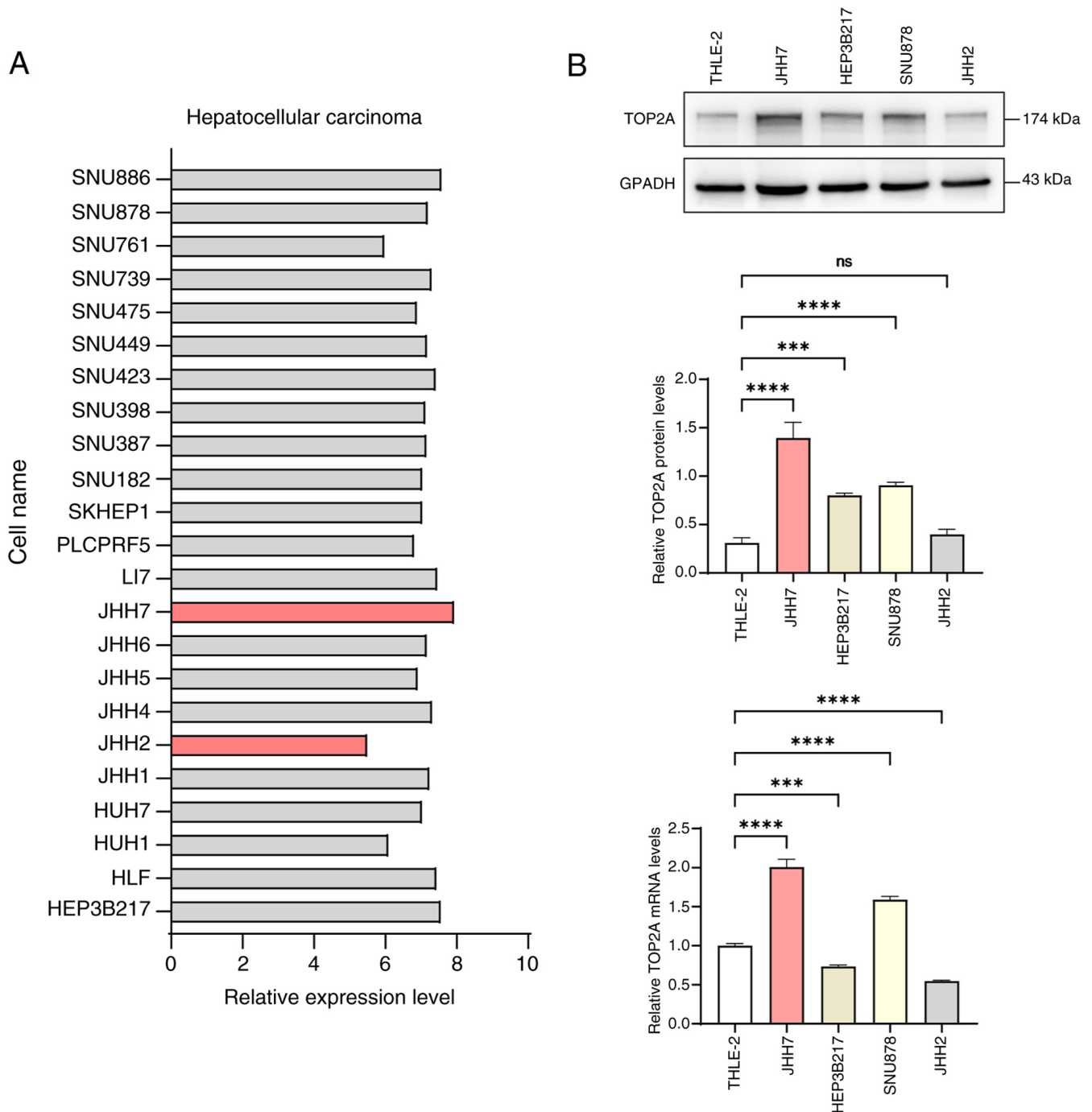


Figure 3. TOP2A expression in different hepatoma cells. (A) Online database (<https://sites.broadinstitute.org/ccle/>) to retrieve different TOP2A expression in liver cancer cells. (B) Western blotting and reverse transcription-quantitative PCR were used to detect protein and mRNA expression levels of TOP2A, respectively, in THLE-2 normal liver cells and liver cancer cells (HEP3B217, SNU878 and JHH2). *** $P < 0.001$ and **** $P < 0.0001$. TOP2A, DNA topoisomerase II alpha; ns, not significant.

of HCC at concentrations of 6.25 $\mu\text{mol/l}$ and 12.5 $\mu\text{mol/l}$. PHN had a strong inhibitory effect on HCC from the concentration interval of 25 to 200 $\mu\text{mol/l}$, accompanied by a concentration dependence. The IC_{50} of PHN-treated JHH7 cells for 24 h was 106.3 $\mu\text{mol/l}$, and that of JHH2 cells was 123.4 $\mu\text{mol/l}$. A PHN concentration of 100 $\mu\text{mol/l}$ ($< \text{IC}_{50}$) was selected for the subsequent experiment (Fig. 4A). To ascertain the impact of PHN on the protein expression of TOP2A, the cell line JHH7 was chosen, known for its high TOP2A expression, and it was subjected to varying

concentrations of PHN for a duration of 48 h. Subsequent analysis revealed that the protein expression of TOP2A was suppressed by PHN in a manner directly proportional to the concentration administered (Fig. 4B).

To further verify the specific role of TOP2A in hepatoma cell lines, TOP2A expression was either knocked down or upregulated. Subsequently, enhanced expression was observed in the JHH7 and JHH2 clones, respectively. Immunoblotting and RT-qPCR analyses revealed that each of the three si-TOP2A constructs significantly suppressed

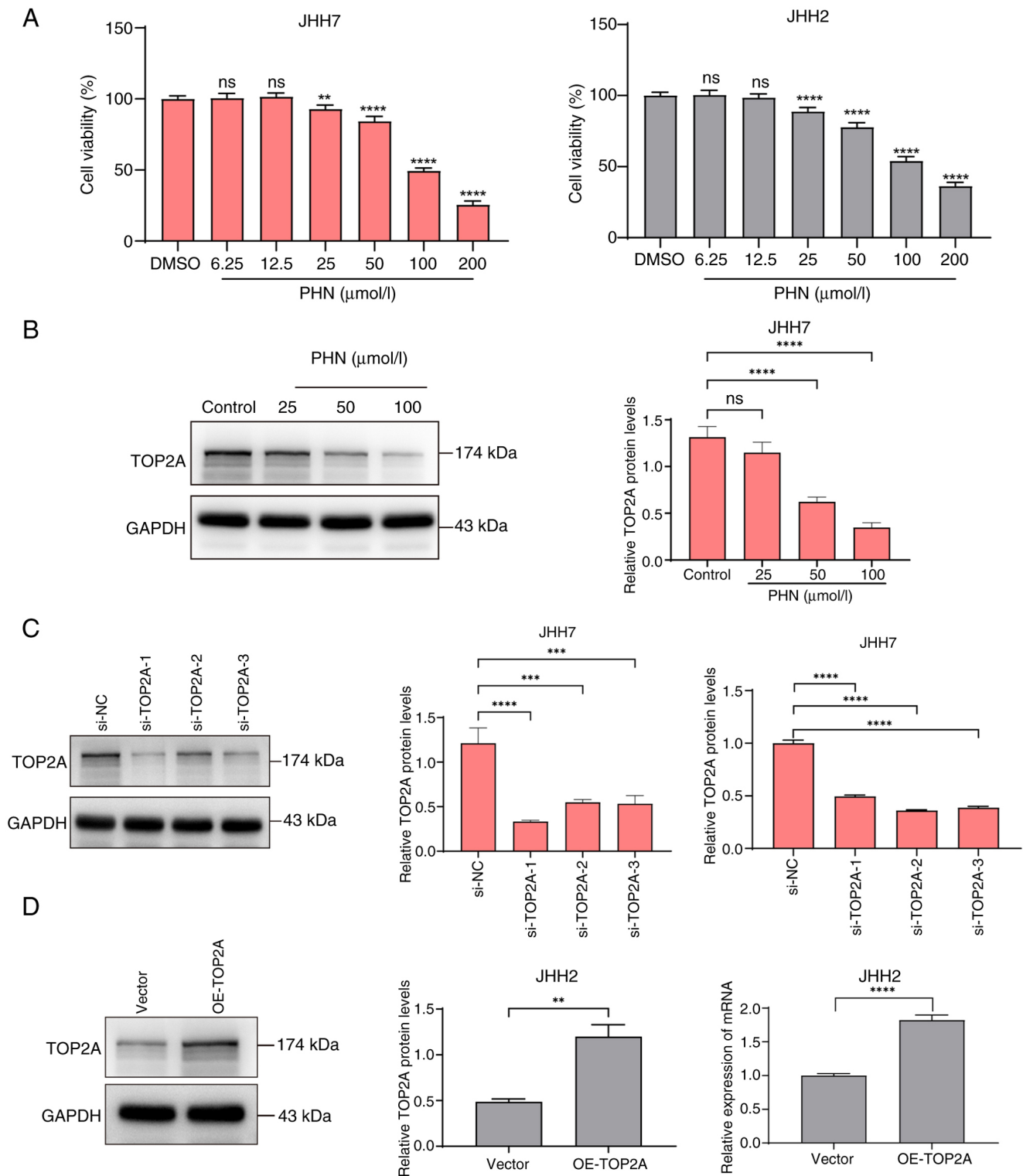


Figure 4. PHN inhibition of hepatocellular carcinoma cell activity and construct a cell model for upregulation and downregulation of TOP2A. (A) The JHH7 and JHH2 cell lines underwent exposure to varying dosages of PHN over a 24-h period, utilizing the Cell Counting Kit-8 method to assess variations in cellular survival rates. (B) Alterations in the TOP2A protein levels within JHH7 cells that had been subjected to diverse PHN concentrations for a duration of 48 h were monitored using WB. (C) Following the introduction of siRNA sequences designed to interfere into JHH7 cells for a 48-h incubation, the protein and mRNA expression levels of TOP2A were evaluated through WB and RT-qPCR, respectively. (D) Post 48-h transfection of JHH2 cells with siRNA and a TOP2A-OE construct, the expression levels of TOP2A protein and mRNA were detected by WB and RT-qPCR. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. PHN, phillyrin; TOP2A, DNA topoisomerase II alpha; WB, western blotting; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR; OE, overexpression; ns, not significant.

the mRNA and protein synthesis of TOP2A (Fig. 2C). Notably, si-TOP2A1 exhibited the most profound suppression of both mRNA and protein synthesis for TOP2A.

Conversely, an upsurge in the mRNA and protein indicators of TOP2A was evident within the OE-TOP2A experimental cohort (Fig. 4D). The experimental results demonstrated

that TOP2A-downregulated cell models in JHH7 cells and TOP2A-upregulated cell models in JHH2 cells were successfully constructed.

CCK-8 and EDU experiments confirmed that PHN group could significantly inhibit the proliferation of JHH7 liver cancer cells compared with the control group, and PHN + si-TOP2A group could inhibit the proliferation of JHH7 liver cancer cells, but there was no statistical significance compared with PHN group. PHN also inhibited the proliferation of JHH2 HCC cells. After PHN treatment, the overexpression of TOP2A plasmid could partially restore the vitality of JHH2 cells. The aforementioned results confirmed that PHN inhibited the proliferation of JHH7 and JHH2 HCC cells by targeting TOP2A (Fig. 5A and B).

PHN can target and inhibit the expression of TOP2A protein to induce ferroptosis of hepatoma cells. To determine the ability of PHN to trigger ferroptosis in hepatoma cells, the changes in mitochondrial morphology and structure were observed by electron microscopy. The electron microscopy structure showed that mitochondria in the control group were oval or rod-shaped, with a double-membrane structure. The outer membrane was smooth, and the inner membrane folded inward to form cristae. However, after treatment with PHN, the outer membrane of mitochondria ruptured, the cristae decreased, and the mitochondrial morphology was disrupted. Nevertheless, overexpression of TO2AP could reverse this phenomenon (Fig. 6A). Attention was also paid to the changes in the intracellular ROS level. The results revealed that PHN treatment could significantly enhance the fluorescence intensity of intracellular ROS, while overexpression of TO2AP could restore the intracellular ROS expression level. In addition, the fluctuations in intracellular Fe^{2+} concentration and MDA content were monitored. The results indicated that PHN significantly increased the intracellular Fe^{2+} concentration and MDA level in hepatoma cells. Furthermore, when PHN was introduced after initially inhibiting TOP2A, it further increased the levels of Fe^{2+} and MDA. Conversely, subsequent overexpression of TOP2A after PHN restored the concentrations of Fe^{2+} and MDA in hepatoma cells to the original levels (Fig. 6B-F). This suggests that PHN can disrupt the mitochondrial structure of hepatoma cells, trigger oxidative stress, increase the expression levels of MDA and Fe^{2+} intracellularly, and promote ferroptosis in hepatoma cells. The mechanism is likely to be achieved by inhibiting the expression level of TOP2A protein.

PHN induces ferroptosis in liver cancer cells by inhibiting the JAK2/STAT3 signaling pathway. To delve deeper into the underlying mechanism by which PHN initiates ferroptosis, the activation of the JAK2/STAT3 signaling axis and the concentration of proteins linked to ferroptotic processes were examined utilizing WB. The findings indicated that PHN significantly inhibited the activation (phosphorylation) of both JAK2 and STAT3, while it had no substantial impact on the overall protein quantities of JAK2 and STAT3. Additionally, PHN was found to decrease the synthesis of glutathione peroxidase 4 (GPX4) and factor inhibiting hypoxia-inducible factor 1 alpha (FIH1), whereas it concurrently enhanced the synthesis of proteins pertinent to ferroptosis, such as COX2,

ACSL4 and NOX1. After TOP2A was downregulated, JHH7 cells were treated with PHN, which enhanced the inhibition of the JAK2/STAT3 signaling pathway. After PHN treatment, overexpression of TOP2A could resist the regulatory effects of PHN on JHH2 cell pathway proteins and ferroptosis-related proteins (Fig. 7A and B). These results suggest that PHN can induce ferroptosis in HCC by inhibiting the JAK2/STAT3 signaling pathway and regulating the expression level of ferroptosis-related proteins.

Discussion

PHN has emerged as a promising therapeutic agent for numerous conditions, owing to its inherent benefits such as minimal toxicity and potent activity, and exhibits remarkable prospects in the realm of cancer treatment (6). Prior to this research, the application of PHN had not been documented in the context of liver carcinoma cells. The groundbreaking aspect of the current investigation lies in uncovering the precise impact of PHN on liver cancer as well as its underlying mode of action. Based on the analysis results of the online database, TOP2A was identified as a potential target of PHN, and the regulatory relationship between PHN and ATP1A3 was further confirmed through molecular docking and WB experiments. The regulatory relationship between PHN and TOP2A has not been reported in the literature. The present results confirmed that PHN can significantly inhibit the expression of TOP2A protein and mRNA. The results of CCK-8 identified that PHN could inhibit the proliferation of JHH2 and JHH7 cells. Interestingly, PHN (100 $\mu\text{mol/l}$) had a survival rate of 49.33% for JHH7 and 54.17% for JHH2. The survival rate of JHH2 cells after PHN treatment is higher, which it was hypothesized that it may be related to the expression level of TOP2A in the cells themselves. In JHH7 cells, the expression level of TOP2A mRNA and protein is higher than that of JHH2 cells, and TOP2A has been proved to promote the process of cancer (25,26). Therefore, PHN has a weaker effect on JHH7 cells than JHH2 cells.

In the prognostic analysis of HCC, TOP2A is considered to be highly correlated with drug resistance and ferroptosis in HCC (27), and similar reports have also been reported in other cancers. TOP2A is a potential biomarker of skin squamous cell carcinoma, and is also highly correlated with genes related to ferroptosis in skin squamous cell carcinoma (28). It is suggested that inhibiting the abnormal expression of TOP2A is a new way to block the progression of cancer. The present results suggested that PHN can downregulate TOP2A and inhibit the progression of liver cancer cells. Ferroptosis is an emerging cellular programmed death mode, which is closely related to HCC (29). Ferroptosis inhibitors GPX4 and FIH1 play a key role in regulating ferroptosis. It has been suggested that the depletion of GPX4 will lead to the accumulation of Fe^{2+} and MDA and eventually induce ferroptosis in HCC cells (30). Inhibition of FIH1 induces ROS accumulation and ultimately leads to ferroptosis in rat myocardial tissue (31). This is consistent with the results of the present study. PHN can induce the increase of the expression levels of MDA, Fe^{2+} and ROS in hepatoma cells, and destroy the mitochondrial structure to induce ferroptosis. It was also found that PHN can inhibit the expression of GPX4 and FIH1 proteins, and activate

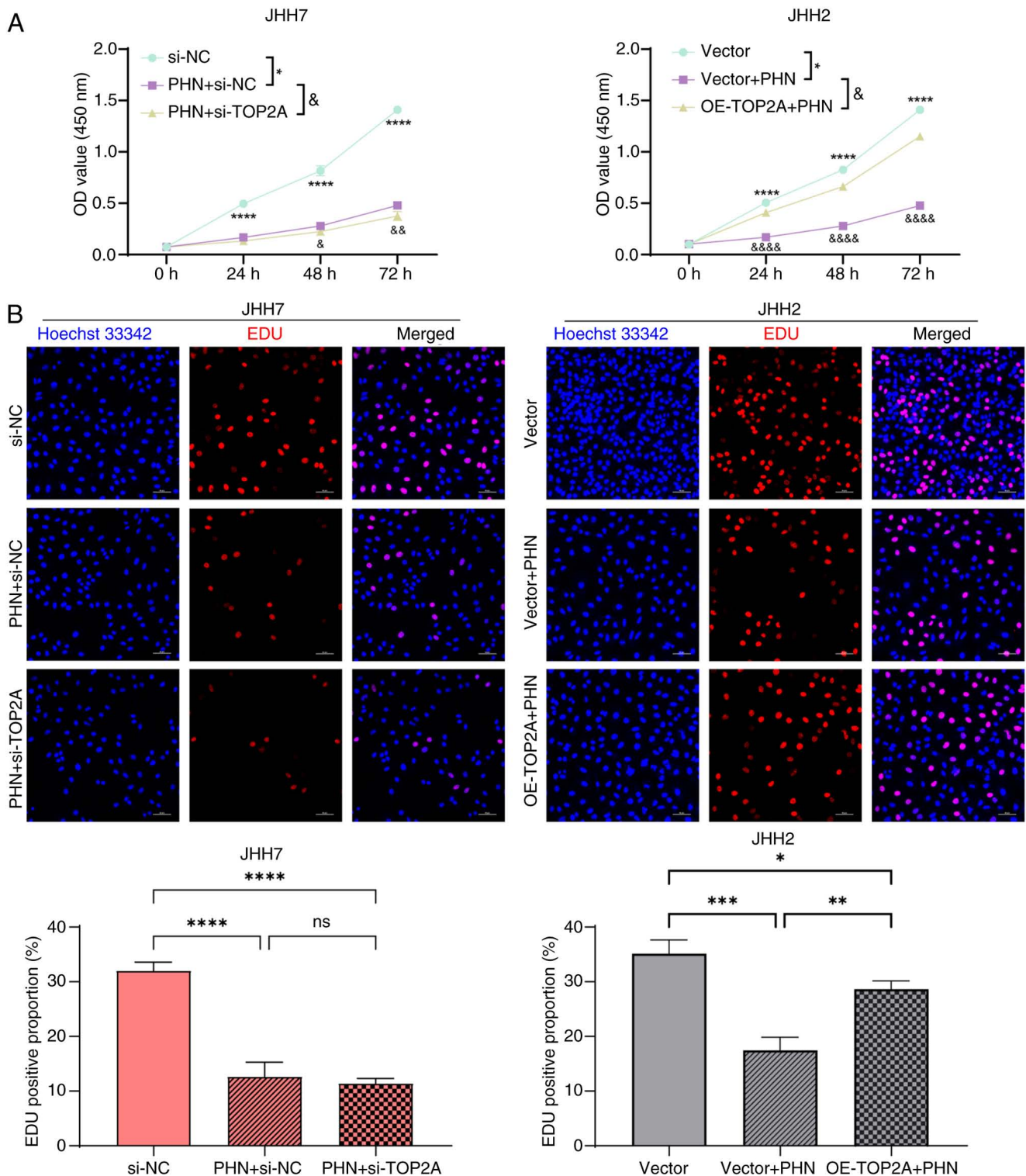


Figure 5. PHN inhibits hepatocellular carcinoma cell proliferation. (A) JHH7 cells pre-treated with PHN for 24 h were selected, and the changes of JHH7 cell viability after transfection with si-TOP2A interference sequence for 24, 48 and 72 h were detected by CCK-8 assay. JHH2 cells transfected with TOP2A overexpression plasmid were selected, and the changes of JHH7 cell viability after PHN treatment for 24, 48 and 72 h were detected by CCK-8 assay. * $P < 0.05$ and **** $P < 0.0001$ between PHN + si-NC and si-NC. & $P < 0.05$, && $P < 0.01$ and &&& $P < 0.0001$ between PHN + si-NC and PHN + si-TOP2A groups. (B) JHH7 cells pre-treated with PHN for 24 h were selected, and the changes of JHH7 cell viability after transfection with si-TOP2A interference sequence for 48 h were detected by EDU assay. JHH2 cells transfected with TOP2A overexpression plasmid were selected, and the changes of JHH7 cell viability after PHN treatment for 24 h were detected by EDU assay. Scale bar, 50 μm . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. PHN, phillyrin; si-, small interfering; TOP2A, DNA topoisomerase II alpha; CCK-8, Cell Counting Kit-8; NC, negative control; ns, not significant.

the expression levels of ACSL4, COX2 and NOX1 proteins. ACSL4, COX2 and NOX1 are all marker proteins that have been previously reported to induce ferroptosis of cells (32-34).

The current results successfully confirmed that PHN induces ferroptosis in liver cancer cells by targeting TOP2A to regulate ferroptosis-related proteins.

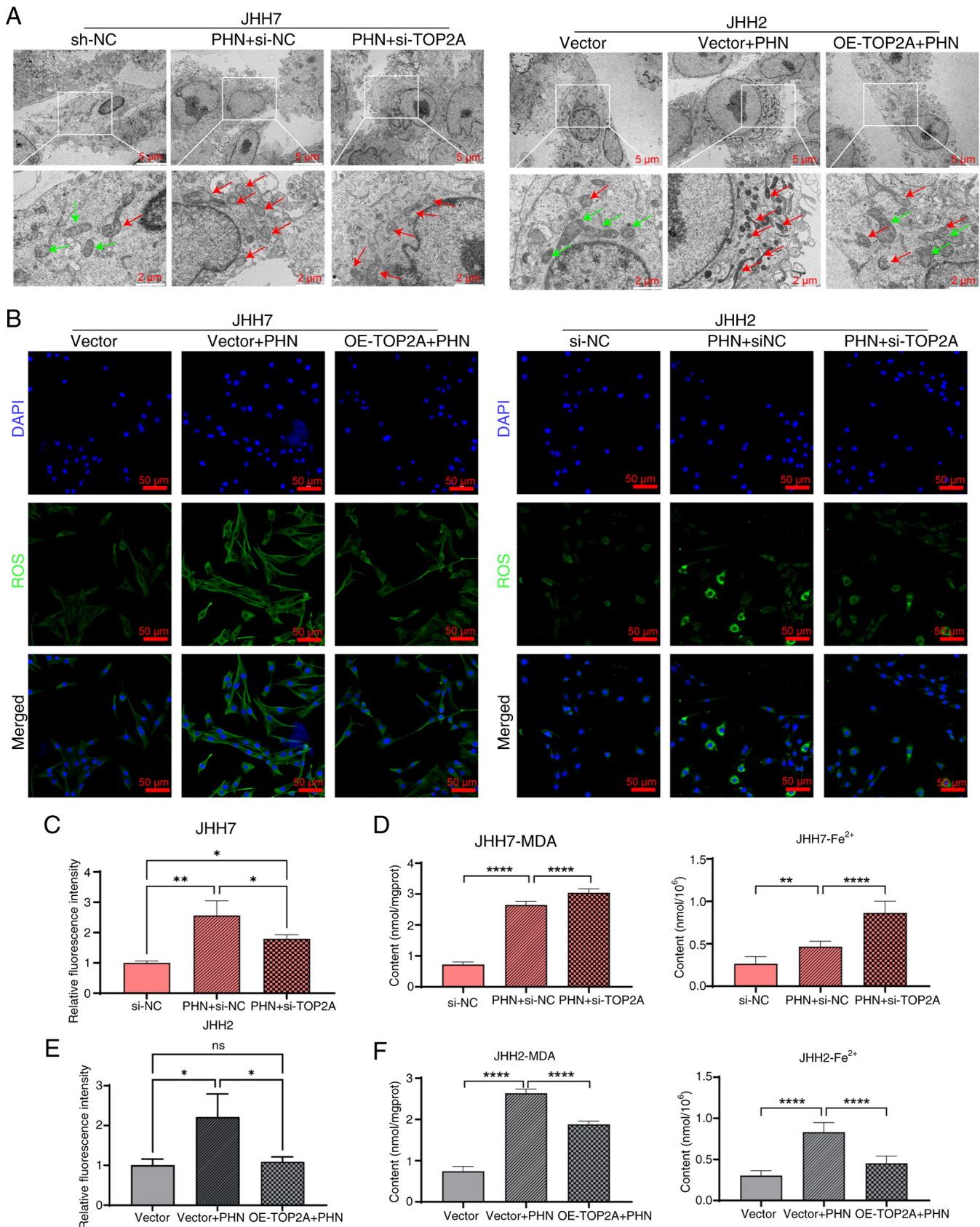


Figure 6. PHN induces ferroptosis in hepatocellular carcinoma cells through inhibition of the JAK2/STAT3 signaling pathway. (A) Changes in the structure and morphology of mitochondria in JHH7 and JHH2 cells after transfection and PHN treatment were observed by electron microscopy. The green arrow points to the normal mitochondrial structure, and the red arrow points to the damaged mitochondrial structure. (B) The changes in intracellular oxidative stress in JHH7 and JHH2 cells after transfection and PHN treatment were detected by ROS staining. (C) Bar graph of relative fluorescence intensity of ROS staining in JHH7 cells. (D) The changes in the levels of MDA and Fe²⁺ were determined in JHH7 cells after treatment according to different grouping requirements. (E) Bar graph of relative fluorescence intensity of ROS staining in JHH2 cells. (F) The changes in the levels of MDA and Fe²⁺ in JHH2 cells were detected after treatment according to different grouping requirements. *P<0.05, **P<0.01 and ****P<0.0001. PHN, phillyrin; ROS, reactive oxygen species; MDA, malondialdehyde; si-, small interfering; OE, overexpression; NC, negative control.

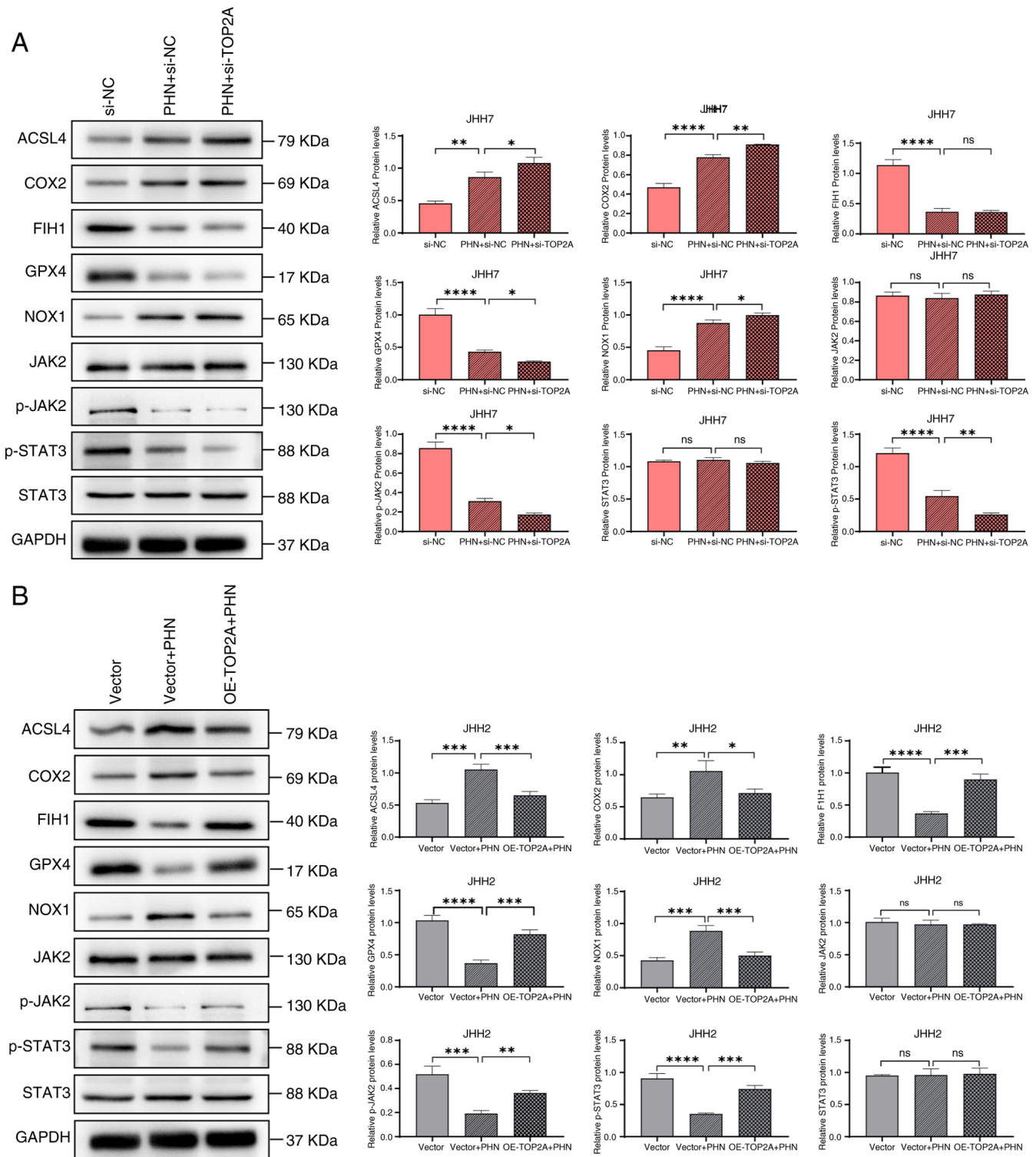


Figure 7. PHN induces ferroptosis in hepatocellular carcinoma cells through inhibition of the JAK2/STAT3 signaling pathway. (A) After treatment with interfering si-TOP2A and PHN, the relative expression levels of ACSL4, COX2, FIH1, GPX4, NOX1, JAK2, p-JAK2, STAT3 and p-STAT3 proteins were detected by WB. (B) After treatment with overexpression plasmids and PHN, the relative expression levels of ACSL4, COX2, FIH1, GPX4, NOX1, JAK2, p-JAK2, STAT3 and p-STAT3 proteins were detected by WB. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. PHN, phillyrin; si-, small interfering; ACSL4, acyl-CoA synthetase family member 4; FIH1, factor inhibiting hypoxia-inducible factor 1 alpha; GPX4, glutathione peroxidase 4; p-, phosphorylated; WB, western blotting; ns, not significant.

The deviant activation of the JAK2/STAT3 signaling cascade has been identified as a detrimental element affecting cancer outcomes (35,36). Recent findings indicate that the engagement of the JAK2/STAT3 signaling route is capable of inducing ferroptotic cell death in breast cancer and bolstering

resistance to therapeutic agents (37). In addition, blocking the activation of the JAK2/STAT3 signaling pathway can induce ferroptosis in cancer cells in osteosarcoma and renal cancer (38,39). The present study emphasizes that PHN can inhibit the expression level of phosphorylated (p-)JAK2 and

p-STAT3 by targeting TOP2A, curtail the function of the JAK2/STAT3 signaling cascade, subsequently stifling the advancement of HCC, aligning with the outcomes of earlier research. Nevertheless, the present findings fail to establish a direct targeting linkage between PHN and the JAK2/STAT3 signaling cascade. The JAK2/STAT3 signaling pathway activator was not added to conduct a reverse verification experiment to further prove our view, which is also the focus of our follow-up work.

In summary, to the best of our knowledge, the present study is the first to demonstrate that PHN can inhibit TOP2A expression in liver cancer cells, block the JAK2/STAT3 signaling pathway and induce ferroptosis in liver cancer cells. While the current findings are encouraging, some questions remain. PHN has low bioavailability, poor water solubility, and requires higher doses to achieve effective concentrations (40,41). The metabolic processes of PHN *in vivo* are not well understood, which may affect its effectiveness and safety (42). In addition, the in-depth mechanism of how PHN inhibits the JAK2/STAT3 pathway and the comprehension of the signaling route remains obscure and necessitates additional validation through the conduct of numerous experimental studies. Therefore, future work will focus on the metabolic pathways of PHN in animals and provide abundant data supporting its therapeutic application. The aim is to solve the problem of the low drug utilization rate of PHN and provide a theoretical basis for the development of its clinical application.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YZ, FH, XL and YH gathered materials, collected data and conducted analyses. YHu was responsible for drafting the initial version of the manuscript. All authors played a pivotal role in the conceptualization and planning of the study, while all participants provided feedback on its earlier iterations. All authors read and approved the final version of the manuscript. YZ and FH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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