

Integrating network pharmacology and experimental validation reveals PI3K/AKT as a critical pathway in pachymic acid-induced inhibition of hepatocellular carcinoma

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ABSTRACT

Pachymic acid (PA), a natural lanost-8-ene-type triterpenoid isolated from *Poria cocos*, exhibits diverse pharmacological activities, including antioxidant, anti-inflammatory, antiaging, and hypnotic effects. Emerging evidence has highlighted its potential anticancer properties, particularly its ability to suppress the proliferation of various cancer cell lines, though the underlying mechanisms remain largely undefined. In this study, we employed a network pharmacology approach to explore the anti-hepatocellular carcinoma (anti-HCC) effects of PA. GO and KEGG enrichment analyses identified the PI3K/AKT signalling pathway as a key candidate mediating PA's activity in liver cancer cells. This prediction was subsequently validated by Western blotting, which confirmed that PA markedly inhibited the growth of HepG2 HCC cells through suppression of the PI3K/AKT pathway, as evidenced by reduced AKT phosphorylation. Collectively, our findings provide new insights into the anticancer mechanisms of PA and support its potential as a promising candidate for novel anti-HCC therapies.

Keywords: network pharmacology, pachymic acid, liver cancer, PI3K/AKT signalling pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. Globally, HCC ranks as the sixth most common cancer and the third leading cause of cancer-related death (1). Its high incidence and mortality rates pose a serious threat to human health. However, current therapeutic options for HCC remain significantly limited. In the early stages of the

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disease, the most effective treatments are often surgical resection of the tumour or liver transplantation. These approaches are frequently associated with a poor prognosis and impose a substantial burden on both patient health and financial resources (2). With advancements in medicine, a range of lower-risk treatment modalities for HCC have emerged. These include targeted therapies, such as epidermal growth factor receptor (EGFR) inhibitors and vascular endothelial growth factor receptor (VEGFR) antagonists (3–5), as well as local ablation therapy for HCC lesions (6), and so forth. Local ablation therapy is characterized by its minimally invasive nature and definitive efficacy, offering a potentially curative opportunity for some HCC patients who are not suitable candidates for surgical resection. Ablation can be performed *via* three primary approaches: percutaneous, laparoscopic, and open surgical. Common ablation modalities include radiofrequency ablation (RFA), microwave ablation (MWA), cryoablation, high-intensity focused ultrasound (HIFU) ablation, and percutaneous ethanol injection (PEI). However, these methods still face challenges such as poor prognosis and high cost (7, 8). To address these issues, there has been a growing shift in focus toward exploring the anticancer potential of traditional Chinese medicine in recent years.

Pachymic acid, derived from the traditional Chinese medicinal fungus *Poria cocos*, is widely recognized for its diuretic, digestive, anticancer, anti-inflammatory, and immune-enhancing properties. Particularly in the realm of anticancer activity, extensive research in recent years has demonstrated that pachymic acid exhibits inhibitory effects against a variety of cancers, including gastric cancer, lung cancer, ovarian cancer, pancreatic cancer, and breast cancer (9–14). Research has demonstrated that in gastric cancer, pachymic acid can inhibit the proliferation and migration of tumour cells by inducing ferroptosis in these cells (15). Concurrently, studies have also revealed that pachymic acid can inhibit the proliferation and migration capabilities of liver cancer cells (16). These findings collectively indicate that pachymic acid possesses a definitive effect in inhibiting tumour cells, holds significant potential in cancer treatment, and demonstrates promise as a novel therapeutic agent for cancer. However, the precise mechanisms underlying the anti-hepatocellular carcinoma effects of pachymic acid remain incompletely understood. The aim of this study was to further investigate the mechanisms by which pachymic acid inhibits hepatocellular carcinoma cells.

EXPERIMENTAL

Cell lines, culture conditions, and chemicals

The HepG2 human hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (ATCC, USA). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere of 5 % CO₂. Pachymic acid was purchased from Chengdu Must Bio-Technology Co., Ltd. (China), with a purity of 99.74 %, and was dissolved in DMSO to prepare a stock solution at a concentration of 9 mmol L⁻¹.

Collection of potential targets of pachymic acid

The potential targets of pachymic acid were systematically collected by integrating multiple public databases. Specifically, targets were directly retrieved using “Pachymic Acid”

from the HERB database. The Comparative Toxicogenomics Database (CTD) was queried with "Pachymic Acid" to gather experimentally validated or predicted targets. The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) was also queried to retrieve known targets of pachymic acid. (Note: OB and DL filters, which are used for screening multi-component compound libraries, were not applied as PA is a single purified compound.) The SwissTargetPrediction database was employed to predict targets based on the SMILES structure of pachymic acid. To ensure prediction reliability, only targets with a probability score > 0.1 were retained, a threshold commonly adopted in network pharmacology studies to balance sensitivity and specificity. Concurrently, the three-dimensional structure of pachymic acid was submitted to the PharmMapper server for pharmacophore matching, and potential targets with a normalized fit score greater than 0.9 were selected. Finally, gene names from all sources were standardized, merged, and deduplicated to construct a comprehensive set of potential targets for pachymic acid.

Collection of hepatocellular carcinoma-related targets

Targets associated with HCC were obtained through a multi-database search. From the GeneCards database, genes with relevance scores above the median were selected after querying "Hepatocellular Carcinoma." The median threshold was chosen as a standard statistical anchor to distinguish genes with substantial evidence from those with weaker associations, thereby balancing sensitivity and specificity. To further validate the robustness of this threshold, we performed a sensitivity analysis using a higher cutoff (relevance score ≥ 10), which confirmed that the PI3K/AKT pathway remained the top enriched hit (data not shown). Using the Online Mendelian Inheritance in Man (OMIM) and Therapeutic Target Database (TTD), known disease-related genes and therapeutic targets were collected by searching with the keywords "Hepatocellular Carcinoma" and "Liver Cancer," respectively. In addition, the dataset GSE216613 (containing HCC and adjacent normal tissues) was downloaded from the Gene Expression Omnibus (GEO) database, and differentially expressed genes were screened with the criteria of an adjusted p -value < 0.05 and $|\log\text{FC}| > 1$ via the GEO2R tool. Targets from all the above sources were merged, duplicates were removed, and a final set of HCC-related targets was constructed.

Screening of "drug-disease" common targets, ppi network construction, and core target analysis

The obtained common targets of "pachymic acid-hepatocellular carcinoma" were submitted to the STRING database to construct a protein-protein interaction (PPI) network, with the species set as "*Homo sapiens*" and a confidence score > 0.4 . The network data were then imported into Cytoscape software for visualization and topological analysis. By calculating topological parameters such as degree, betweenness centrality, and closeness centrality for each node in the network, key nodes whose values were all above the median were identified and determined as potential core targets for the anti-HCC activity of pachymic acid.

GO and KEGG enrichment analysis

To elucidate the biological functions and signalling pathways associated with the common targets, the list of "pachymic acid-hepatocellular carcinoma" common targets was

submitted to the SRplot platform (bioinformatics.com.cn) (17) for enrichment analysis. Both Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed. The SRplot platform integrates clusterProfiler, which uses Fisher's exact test as the default statistical method and the human genome as the background gene set; multiple testing correction was performed using the Benjamini-Hochberg (BH) method to control the false discovery rate. The GO analysis covered three major categories: biological process, cellular component, and molecular function. A p -value < 0.05 was set as the threshold for statistical significance. The top-ranked enriched terms were screened and visualized to systematically reveal their potential biological functions and pathway mechanisms.

Cell viability assay

After cell culture, an equal number of HepG2 cells were seeded into each well of a 96-well plate. Following cell attachment, PA was diluted into a series of concentration gradients and added to the plate in equal volumes. After the designated drug treatment period, 100 μ L of the CCK-8 reagent (Beijing Lanjie Ke Technology Co., Ltd., China) was added to each well at a 10 % final concentration. The plate was then incubated, and the absorbance of each well was measured using a microplate reader.

Wound healing experiment

Cells were seeded into 6-well plates. Upon reaching approximately 80 % confluence, straight scratches were made across the cell monolayer using sterile 1000 μ L pipette tips. The cells were subsequently incubated in medium for 48 hours. Wound closure was monitored under an inverted microscope. The migration and healing ability were quantitatively assessed by measuring the wound area with ImageJ software.

Protein extraction and Western blotting

Following a wash with phosphate-buffered saline (PBS), total cellular protein was isolated using RIPA lysis buffer. The buffer contained a cocktail of protease and phosphatase inhibitors (purchased from Beyotime). Protein concentration was determined with a bicinchoninic acid (BCA) assay kit (Beijing Lanjie Ke Technology Co., Ltd.).

Following sonication and centrifugation, the supernatant was harvested, and its protein concentration was determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad, USA). Equal amounts of protein (30 μ g) from each sample were denatured at 95 °C for 5 min, resolved by SDS-PAGE, and subsequently transferred to a nitrocellulose membrane (Bio-Rad) using a Mini-PROTEAN system (Bio-Rad). The membrane was blocked with 5 % fetal bovine serum (FBS) for 1 h at room temperature, then incubated overnight at 4 °C with the following primary antibodies: anti-AKT (Cell Signaling Technology, USA, Cat# 9272S, dilution 1:1000), anti-phospho-AKT (Ser473) (Cell Signaling Technology, Cat# 4060S, dilution 1:1000), and anti- β -actin (Cell Signaling Technology, Cat# 4967S, dilution 1:2000). After washing, the membrane was incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad, Cat# 1706515, dilution 1:5000) for 1 h at room temperature. Finally, after treatment with Clarity Western

ECL Substrate (Bio-Rad), signals were detected using an imaging system (Azure Biosystems, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., USA). Data are presented as the mean $\pm$ SD. For the CCK-8 assay, one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to compare each PA-treated group with the control group. For the wound healing assay and Western blot analysis, one-way ANOVA followed by Tukey's post-hoc test was performed for multiple pairwise comparisons among all groups. A p -value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Target screening results

Through multi-database retrieval, a total of 167 potential targets of pachymic acid were obtained. Concurrently, 12,777 hepatocellular carcinoma-related targets were collected from disease databases. After intersecting the two sets, 148 common targets of "pachymic acid-hepatocellular carcinoma" were identified (Fig. 1). These targets represent the potential basis for the intervention of pachymic acid in hepatocellular carcinoma.

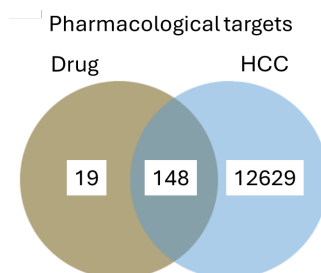


Fig. 1. Intersection analysis of pharmacological targets of pachymic acid and disease targets of hepatocellular carcinoma.

PPI network construction and core target analysis

The PPI network constructed from the 148 common targets was imported into Cytoscape for visualization. The network comprised 148 nodes and 453 interaction edges, exhibiting a moderately high density that suggests extensive functional associations among the targets (Fig. 2a). The top 10 targets with the highest degree values in the PPI network were identified as core targets, including EGFR, BCL2, MAPK1, SRC, FN1, ALB, RELA, CASP3, ESR1, and PTGS2, all of which play crucial roles in tumorigenesis and progression (Fig. 2b).

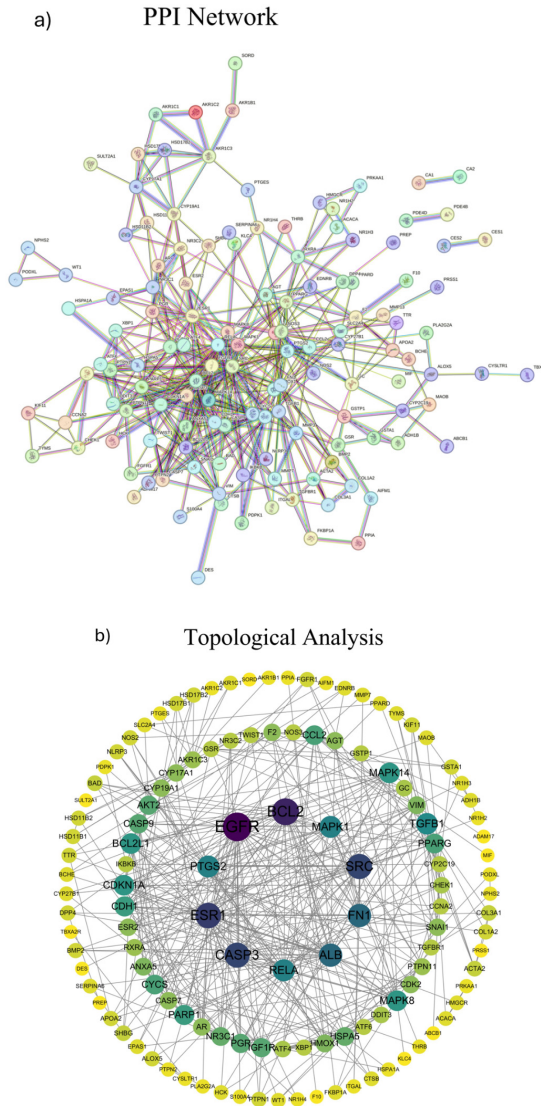


Fig. 2. a) Analysis and construction of a PPI network using common targets of pachymic acid and hepatocellular carcinoma; b) core targets identified through topological analysis of the PPI network.

GO and KEGG enrichment analysis results

GO and KEGG enrichment analyses conducted *via* the SRplot platform (bioinformatics.com.cn) systematically revealed the potential functions and pathways of the common targets.

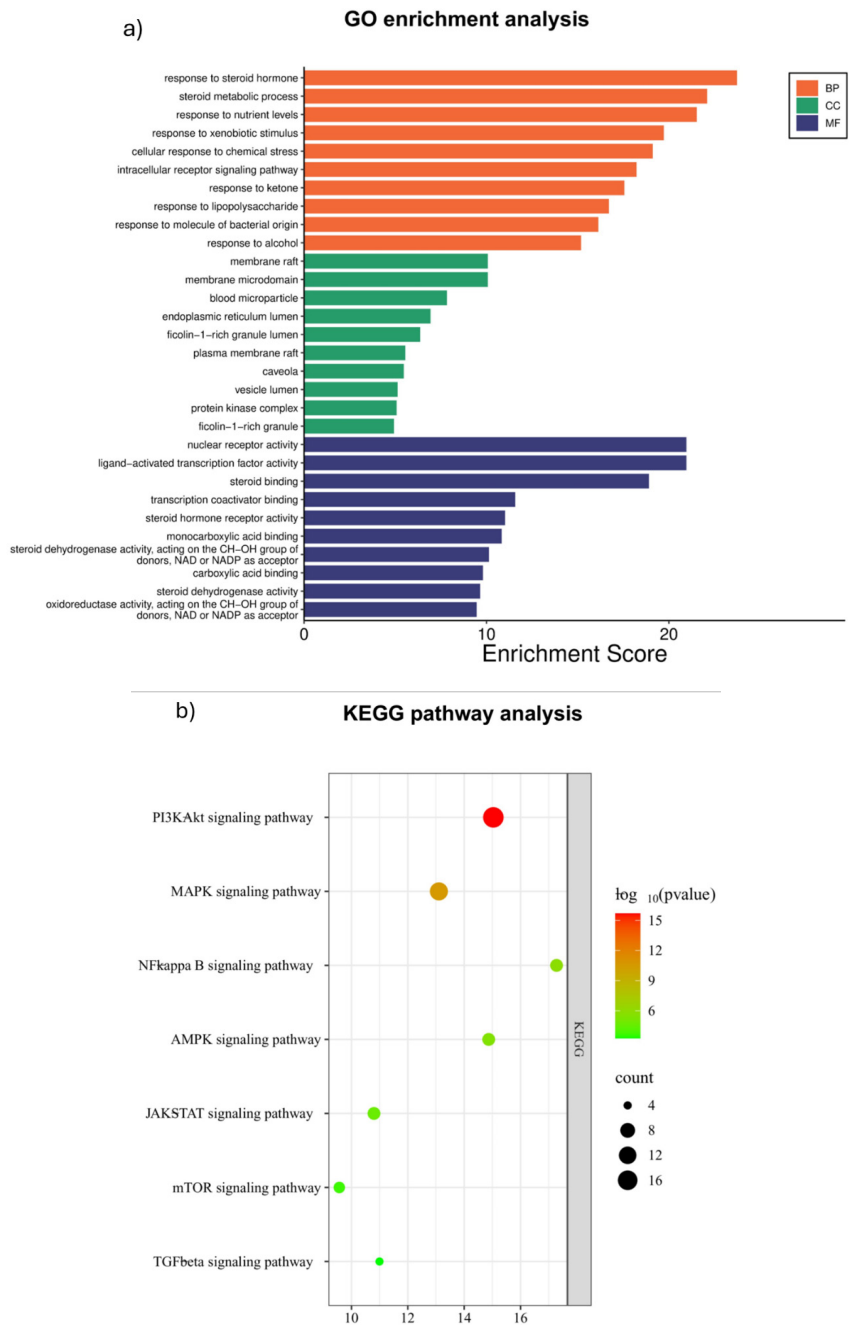


Fig. 3. a) GO enrichment analysis of common targets of pachymic acid and hepatocellular carcinoma; b) KEGG enrichment analysis of common targets of pachymic acid and hepatocellular carcinoma.

GO analysis indicated that the targets were significantly enriched in biological processes such as the regulation of cell signalling pathways and hormone metabolism. They were primarily localized in the membrane microdomains, plasma membrane rafts, and the endoplasmic reticulum lumen, with molecular functions involving nuclear receptor activity, steroid binding, and transcription factor activity (Fig. 3a). KEGG pathway analysis further demonstrated that these targets were highly enriched in key pathways closely associated with tumorigenesis and progression, including the PI3K/AKT signalling pathway, the MAPK signalling pathway, and the AMPK signalling pathway (Fig. 3b). These findings suggest that pachymic acid may exert its anti-HCC effects by modulating this multifaceted functional and signalling network.

PA inhibits the proliferation of HepG2 liver cancer cells

To assess the effect of PA on liver cancer cells, we seeded HepG2 cells into 96-well plates at a density of 6,000 cells per well.

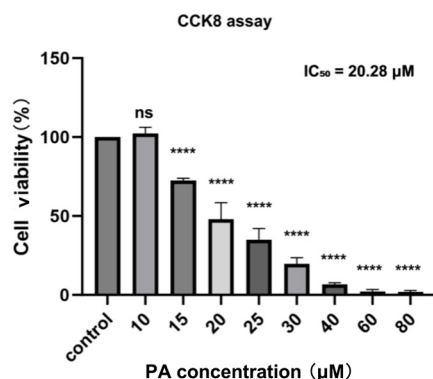


Fig. 4. Inhibitory effect of PA on HepG2 cell viability assessed by the CCK-8 assay after 48 h of treatment. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for comparisons between each PA-treated group and the control group. **** $p < 0.0001$. $IC_{50} = 20.28 \mu\text{mol L}^{-1}$.

PA was diluted into a series of concentration gradients (0, 10, 15, 20, 25, 30, 40, 60, and $80 \mu\text{mol L}^{-1}$) and added to the respective wells. After 48 h of drug treatment, $100 \mu\text{L}$ of CCK-8 reagent (at a final ratio of 10 %) was added to each well, and the absorbance was measured at 450 nm using a microplate reader. The resulting IC_{50} value was $20.28 \mu\text{mol L}^{-1}$.

PA significantly inhibits the migration of HepG2 cells

To investigate the effect of PA on the migratory ability of HepG2 cells, a wound healing assay was conducted.

Cells were treated with different concentrations of PA and grouped accordingly. The wound area in each group was measured using ImageJ software. Statistical significance was

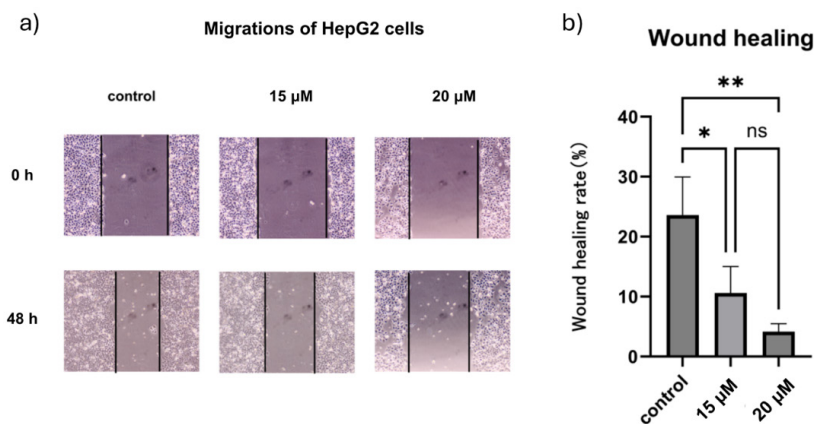


Fig. 5. a) PA inhibits the migration of HepG2 cells (48 h); b) quantification of wound closure rates. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using one-way ANOVA with Tukey's test for comparisons among all groups. * $p < 0.05$, ** $p < 0.01$.

determined using one-way ANOVA followed by post-hoc pairwise comparisons. The differences between the control group and both PA-treated groups were statistically significant ($p < 0.05$). In our study, PA effectively reduced the migratory capacity of HepG2 cells.

PA suppresses the phosphorylation of the AKT protein within the PI3K/AKT signalling pathway in HepG2 cells

To verify whether PA inhibits the PI3K/AKT pathway in liver cancer cells, we assessed the phosphorylation level of AKT protein in PA-treated HepG2 cells by Western blot analysis. The results are shown in Fig. 6. After 48 h of treatment with 20 $\mu\text{mol L}^{-1}$ PA, the

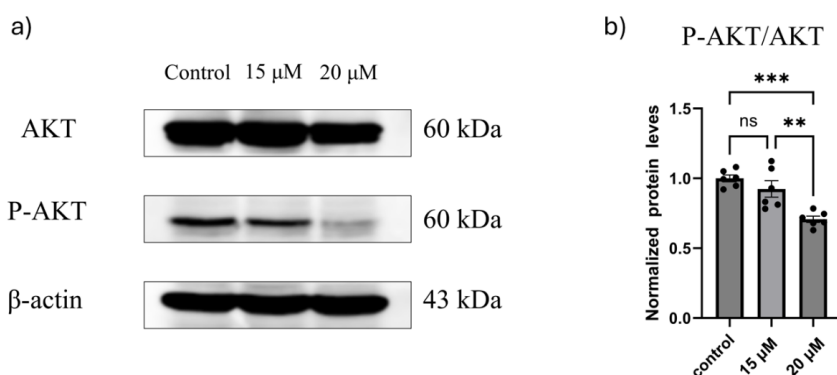


Fig. 6. a) Western blot analysis of AKT phosphorylation in control and PA-treated groups; b) quantification of p-AKT protein levels relative to total AKT by densitometric analysis of Western blot bands. Statistical significance was determined using one-way ANOVA with Tukey's test for comparisons among all groups. ** $p < 0.01$, *** $p < 0.001$.

expression level of total AKT protein in the experimental group showed little difference compared with the control group, whereas a marked reduction was observed in the relative expression of phosphorylated AKT (p-AKT). Densitometric analysis of the Western blot bands revealed significantly lower p-AKT band intensity in the PA-treated group than in the control, indicating that the phosphorylation of AKT was suppressed by PA treatment.

Discussion

HCC ranks among the most formidable malignancies threatening human health, notorious for its high mortality and incidence rates. Due to its intricate and not yet fully elucidated pathogenesis, effective pharmacological interventions remain limited. While surgical resection is a primary treatment modality, it is often associated with a poor prognosis. Consequently, the current therapeutic landscape for HCC faces significant limitations. Hence, the search for novel therapeutic agents against hepatocellular carcinoma represents a crucial mission for medical researchers. Given the significant advantages of traditional Chinese medicine, such as superior prognosis and milder therapeutic effects, a growing number of research teams have consequently shifted their focus to the active components of Chinese herbal medicines. Notably, pachymic acid, a natural compound derived from *Poria cocos*, has come into research focus due to its demonstrated anticancer activity against multiple cancer types.

In recent years, research teams have discovered that pachymic acid exerts anticancer effects through diverse mechanisms across various cancer types. For instance, in endometrial cancer, it degrades nuclear receptor subfamily 3 group C member 1 (NR3C1) and inhibits cell migration by suppressing the NR3C1/TGF- β /SMAD signalling pathway (9); in cervical cancer, it induces endoplasmic reticulum stress, causes mitochondrial dysfunction, and activates the AMPK pathway to exert antitumour activity (18). It also modulates the tumour cell cycle by inhibiting the formation of cyclin D-CDK 4/6 and cyclin E-CDK 2 complexes, leading to cell-cycle arrest at the G0/G1 phase (19). Furthermore, in the field of combination therapy, pachymic acid also demonstrates unique advantages. Research has shown that the combination of PA and bavachin can enhance the metabolic stability of bavachin, inhibit its transport within lung cancer tumour cells, and effectively potentiate bavachin's inhibitory effect on lung cancer cells (20). In addition to interacting with other drugs, PA can also be combined with other treatment modalities to enhance therapeutic efficacy. For example, Lu *et al.* (21) found that PA can upregulate the expression of Bax by inhibiting HIF1, thereby increasing the sensitivity of gastric cancer cells to radiation and improving the efficacy of radiotherapy. These examples sufficiently demonstrate the significant potential of PA to become an effective anticancer agent.

Network pharmacology is an interdisciplinary research paradigm that integrates systems biology, bioinformatics, and polypharmacology. Its core lies in constructing complex interaction networks among drugs, targets, and diseases to explain, from a systemic perspective, how drugs intervene in disease networks. This method overcomes the limitations of the traditional drug research model focused solely on single targets (22). It is particularly well-suited for analysing the synergistic principles of multiple targets and pathways in traditional Chinese medicines and their active components, thereby providing a crucial technical pathway for the modernization of Chinese medicine research. Network pharma-

cology has now become a reliable method for studying the mechanisms of action of pharmacological components and their effects on diseases. A growing number of researchers utilize network pharmacology as a guidance and theoretical foundation, followed by validation through cellular or animal experiments (8, 23). Network pharmacology has emerged as a powerful tool for predicting the multi-target mechanisms of natural products. In the present study, we employed this approach to investigate the anti-HCC effects of PA. Through multi-database screening, we identified 148 common targets between PA and HCC. GO and KEGG enrichment analyses revealed that these targets were significantly enriched in several cancer-associated pathways, particularly the PI3K/AKT signalling pathway. The GO analysis indicated that the common targets were primarily involved in steroid metabolism and responses to steroid hormones and xenobiotic stimuli, pathways closely associated with liver metabolic function and implicated in hepatocarcinogenesis. In terms of cellular components, these targets were mainly localized in membrane microdomains, the plasma membrane rafts, and the endoplasmic reticulum lumen; lipid rafts and caveolae are known to serve as signalling platforms for various oncogenic pathways in HCC. The enriched molecular functions included nuclear receptor activity, steroid binding, and transcription factor activity, consistent with the role of nuclear receptors in regulating hepatic metabolism and HCC progression. Given the well-established role of the PI3K/AKT pathway in HCC progression – where it promotes tumour cell proliferation, migration, and angiogenesis – we focused on validating this pathway experimentally.

The PI3K/AKT pathway is a well-recognized signalling pathway implicated in hepatocellular carcinoma. Research has demonstrated that within the hepatocellular carcinoma tumour microenvironment, ITIH2 secreted by liver tumour cells can activate the PI3K/AKT signalling pathway through ubiquitin-mediated stabilization of THBS1, thereby enhancing the angiogenic capacity of tumour endothelial cells and promoting the progression of hepatocellular carcinoma (24). Furthermore, several tumour-associated regulatory factors are also modulated by the PI3K/AKT signalling pathway, thereby influencing the progression of hepatocellular carcinoma. As exemplified by CGREF1 (25), a recently identified secreted protein linked to HCC progression, it has been shown to suppress autophagy *via* the PI3K/AKT pathway, which in turn enhances the proliferation and migration of liver cancer cells and promotes tumour development (26). In the field of pharmacology, numerous agents exert antitumor effects by inhibiting the PI3K/AKT signalling pathway. For instance, sea buckthorn has been shown to alleviate oxidative stress in HepG2 cells by scavenging free radicals, suppressing pro-inflammatory cytokines, and modulating the PI3K/Akt and JAK pathways, thereby inhibiting hepatocellular carcinoma cells and demonstrating potential for further development as a therapeutic candidate for liver cancer (27).

To test the hypothesis generated by the network pharmacology analysis, we performed a series of *in vitro* experiments focusing specifically on the top-ranked PI3K/AKT pathway. CCK-8 assays confirmed that PA significantly inhibited HepG2 cell viability with an IC_{50} of approximately $20.28 \mu\text{mol L}^{-1}$. Notably, a previous study reported an IC_{50} of approximately $41.07 \mu\text{mol L}^{-1}$ for PA against HepG2 cells after 48 h of treatment (16). We attribute this discrepancy to inter-laboratory variability, including differences in culture reagents, cell stocks, and consumables, as our repeated experiments consistently yielded IC_{50} values around $20 \mu\text{mol L}^{-1}$ with appropriate DMSO controls. However, both sets of findings consistently indicate that PA exerts a marked inhibitory effect on HepG2 cells. Wound healing assays demonstrated that PA significantly inhibited HepG2 cell migration

at the tested concentrations. Western blot analysis further revealed that PA suppressed AKT phosphorylation without affecting total AKT expression, confirming that the PI3K/AKT pathway mediates the anti-HCC effects of PA. While the inhibitory effects of PA on HCC cell viability and migration have been previously reported, the demonstration that PA exerts these effects specifically through suppression of the PI3K/AKT pathway *via* reduced AKT phosphorylation constitutes the primary mechanistic contribution of this study.

The innovation of this study lies in the integration of network pharmacology with experimental validation to identify the correlation between the PI3K/AKT pathway and PA-induced inhibition of hepatocellular carcinoma. This integrated strategy represents a well-established and effective approach for elucidating the molecular mechanisms of natural products, and our study provides a practical example of its application to pachymic acid.

Nevertheless, this study employed network pharmacology as the theoretical framework and validated the findings through cellular experiments with subsequent in-depth analysis. It has not yet incorporated animal studies to provide multidimensional verification. Future research could be extended based on these findings.

Furthermore, while the PPI network analysis identified 10 core targets (EGFR, BCL2, MAPK1, SRC, FN1, ALB, RELA, CASP3, ESR1, and PTGS2), the present study focused on validating the PI3K/AKT pathway rather than these individual targets. The drug-disease common targets identified *via* network pharmacology in this work remain largely unexplored experimentally, and we acknowledge this as a limitation. Future studies – including target-specific binding assays, gene knockdown/overexpression experiments, and animal models – are warranted to systematically investigate whether PA directly interacts with these core targets and how such interactions contribute to its anti-HCC activity.

CONCLUSIONS

Employing network pharmacology, this study analysed the relationship between pachymic acid (PA) and hepatocellular carcinoma. Multiple common targets and associated pathways between the drug and the disease were identified through computational screening. Among these, the PI3K/AKT pathway was selected for experimental validation, and the results confirmed that PA suppresses AKT phosphorylation in HepG2 cells, indicating that the anti-HCC effect of PA is at least partially mediated through inhibition of the PI3K/AKT signalling pathway. This work provides preclinical evidence supporting the potential of PA in the treatment of hepatocellular carcinoma.

Data availability statement. – All data and figures presented in this study are original and authentic. Further details are available from the corresponding author upon reasonable request.

Ethics statement. – The cell lines used in this study were commercially purchased. According to local regulations and institutional policies, no ethical approval was required.

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Conflict of interest. – The authors declare that there are no potential conflicts of interest regarding this study.

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Author's contribution. – Conceptualization, M.T., Z.P., and T.S.; methodology, J.L. and H.Z.; investigation, J.L., H.Z., and S.P.; resources, M.T. and Z.P.; data curation, J.L. and H.Z.; visualization, J.L. and H.Z.; supervision, M.T. and Z.P.; writing, original draft preparation, J.L.; writing, review and editing, Z.P., T.S., and M.T.; project administration, Z.P.; funding acquisition, M.T. All authors have read and approved the final version of the manuscript.

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