Network Pharmacology-Based Investigation of Wogonin's Mechanism of Action Against Hepatocellular Carcinoma and In Vitro Experimental Study: Postprint

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Abstract

Background: Hepatocellular carcinoma is a leading cause of cancer-related mortality, and the current prevention and treatment landscape remains challenging. Investigating novel therapeutic agents for hepatocellular carcinoma holds scientific significance.

Objective: To analyze the mechanism of wogonin intervention in hepatocellular carcinoma through network pharmacology approaches and validate the findings through in vitro experiments.

Methods: Drug targets of wogonin were retrieved from the TCMSP database, while disease targets for hepatocellular carcinoma were collected from the TTD, GenCard, OMIM, and DisGent databases. The collected drug targets and disease targets were intersected to obtain potential targets for drug intervention in the disease. Enrichment analysis of the intersecting targets was performed using R software, and protein-protein interaction networks were constructed and core targets were screened using the STRING database and Cytoscape software. The core targets were further analyzed in the GIEPA database. Finally, the preliminary analysis results were validated through in vitro experiments: cell viability was measured using the CCK-8 assay kit; cell proliferation was assessed using the plate colony formation assay; cell migration was evaluated using the scratch assay; and protein expression levels were determined using Western blotting (WB) experiments.

Results: The analysis revealed that the AMDE properties of wogonin conform to the druggability rules for small-molecule drugs, and toxicity analysis indicated no toxicity. A total of 135 wogonin targets and 8,238 hepatocellular carcinoma targets were collected, yielding 113 intersecting targets. Analysis of the TOP10

core genes screened from the constructed protein-protein interaction network revealed that CDK1 and SRC were upregulated at the mRNA level in hepatocellular carcinoma tissues compared to normal liver tissues (P<0.05), and their high expression was associated with poor prognosis in hepatocellular carcinoma patients (P<0.05). KEGG enrichment analysis demonstrated that the intersecting genes were most significantly enriched in the PI3K/AKT signaling pathway, and molecular docking results showed that wogonin exhibited strong binding affinity to CDK1 and SRC. CCK-8 assay results showed that HepG2 cell viability in groups treated with 75, 150, and 300.0 mol/L wogonin was significantly lower than that in the control group (P<0.05). Plate colony formation assay results indicated that the number of colonies formed by HepG2 cells in groups treated with 37.5, 75, and 150.0 mol/L wogonin was significantly lower than that in the control group (P<0.05). Scratch assay results demonstrated that the migration rate of HepG2 cells in groups treated with 37.5, 75, and 150.0 mol/L wogonin was significantly lower than that in the control group (P<0.05). WB experimental results revealed that the protein expression levels of PI3K, P-AKT/AKT, CDK1, and SRC in groups treated with 75 and 150.0 mol/L wogonin were significantly lower than those in the control group (P<0.05).

Conclusion: Wogonin intervenes in the occurrence and progression of hepatocellular carcinoma by downregulating CDK1 and SRC protein expression, attenuating PI3K/AKT pathway signaling, inhibiting hepatocellular carcinoma cell proliferation and migration, and inducing apoptosis.

Full Text

Mechanism and in Vitro Experiment of Wogonin in Treatment of Hepatocellular Carcinoma Based on Network Pharmacology

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Abstract

Background

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related deaths. The current prevention and treatment situation remains critical. It is of scientific significance to explore new therapeutic agents for HCC.

Objective
To analyze the mechanism of wogonin on HCC by network pharmacology and to verify it in vitro.

Methods
The drug targets of wogonin were searched in TCMSP database, and the disease targets of HCC were collected from TTD, GenCard, OMIM, DisGent databases. The collected drug targets and disease targets were intersected as potential targets for drug intervention in diseases. R software was used for enrichment analysis of intersection targets, STRING database and Cytoscape software were used to construct protein interaction network and screen core targets. The core targets were further analyzed in GIEPA database. Finally, the preliminary analysis results were verified by in vitro experiments, including cell activity determination using CCK-8 kit, cell proliferation determination using plate clone formation experiment, cell migration determination using scoring test, protein expression level determination using Western-blotting (WB) assay.

Results
The AMDE characteristics of wogonin were found to be in accordance with the rules for small molecule drug formation and the toxicity analysis showed no toxicity. A total of 135 wogonin targets and 8238 HCC targets were collected, and 113 targets were intersected. Through the analysis of the core genes of TOP10 screened by the constructed protein interaction network, it was found that the mRNA levels of CDK1 and SRC in liver cancer tissues were higher than those in normal liver tissues ($P<0.05$), and the high expression levels in liver cancer patients were related to poor prognosis ($P<0.05$). KEGG enrichment analysis showed that the intersection genes were enriched in the PI3K/AKT signaling pathway, and the molecular docking results showed that wogonin had strong binding configuration activity with CDK1 and SRC. The results of CCK-8 kit showed that the activity of HepG2 cells in the 75, 150, and 300.0 mol/L wogonin groups was lower than that in the control group ($P<0.05$). The results of plate clone formation experiment showed that the number of colony formation of HepG2 cells in the 37.5, 75, 150.0 mol/L wogonin groups was lower than that in the control group ($P<0.05$). The results of scoring test showed that the migration rate of HepG2 cells in the 37.5, 75 and 150.0 mol/L wogonin groups was lower than that in the control group ($P<0.05$). The results of the WB assay showed that the expression levels of PI3K, P-AKT/AKT, CDK1 and SRC proteins in the 75 and 150.0 mol/L wogonin groups were lower than those in the control group ($P<0.05$).

Conclusion
Wogonin inhibits the proliferation and migration of HCC cells and induces apoptosis by down-regulating the expression of CDK1 and SRC proteins and attenuating the PI3K/AKT pathway signaling, to achieve the purpose of interfering with the occurrence and progression of HCC.

Keywords
Carcinoma, hepatocellular; Wogonin; Network pharmacology; In vitro experi-

ments

Introduction

Hepatocellular carcinoma (HCC) accounts for the majority of primary liver cancers and is a leading cause of cancer-related mortality worldwide [1]. Sorafenib and lenvatinib have been approved as first-line therapeutic agents for HCC in most Asian countries, the United States, and the European Union [2]. Over the past two decades, clinical management of HCC has been extensively investigated, leading to significant improvements in treatment regimens, including novel drug combinations. However, despite considerable progress, overall outcomes for HCC remain far from satisfactory [3]. Therefore, exploring new therapeutic approaches for HCC is of significant scientific importance. Pharmacotherapy represents a crucial component of cancer treatment [4-5]. In drug development, lead compound screening in preclinical studies plays a vital role [6-7]. Plants serve as an important resource for lead compounds in drug discovery because they produce numerous pharmacologically active metabolites [8]. However, conventional lead compound screening methods are characterized by long cycles, heavy workload, and high costs [9-10]. The development of network pharmacology has opened new avenues for screening lead compounds from active ingredients in medicinal plants [11]. Wogonin is a flavonoid natural compound and the main active component of Scutellaria plants [12]. Previous studies have demonstrated that wogonin possesses promising antitumor activity [13]. This study aims to analyze the mechanism of wogonin in treating HCC through network pharmacology and validate the findings through in vitro experiments, hoping to explore new therapeutic options for HCC.

Materials and Methods

1.1 Prediction of ADME Properties and Toxicity of Wogonin

The SMILES formula of wogonin was retrieved from the PubChem database. The retrieved SMILES was imported into the SWISS ADME online service platform (http://www.swissadme.ch/) to analyze the absorption, distribution, metabolism, excretion (ADME) properties and bioavailability of wogonin. The ProTox-II-Prediction of Toxicity of Chemicals online service platform (https://tox-new.charite.de/protox_{II}/index.php?site=compound_{input}) was used to predict the hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, and cytotoxicity of wogonin.

1.2 Collection of Wogonin and Hepatocellular Carcinoma Targets

The TCMSP database (https://tcmsp-e.com/tcmsp.php) was searched using the keyword “wogonin” to collect wogonin drug targets. Using the keyword “hepatocellular carcinoma,” HCC disease targets were retrieved and collected from the

TTD (https://db.idrblab.net/ttd/), GeneCards (https://www.genecards.org/), OMIM (https://www.omim.org/), and DisGeNET (https://www.disgenet.org/) databases. The intersection of drug targets and disease targets was taken to obtain potential targets for drug intervention in disease.

1.3 Protein-Protein Interaction Network Construction and Core Target Screening

The potential targets for drug intervention in disease were imported into the STRING database (https://cn.string-db.org/), with species selected as "Homo sapiens" and required score set to "highest confidence (0.900)" to construct a protein-protein interaction network. The protein-protein interaction network was imported into Cytoscape 3.9.0 software (hiding nodes with degree of 0), and the "Cytohubba" plugin was used to screen the TOP 10 genes. The GEPIA database (http://gepia2.cancer-pku.cn/#index) was then used to analyze the mRNA expression levels of core genes in liver cancer tissues and normal liver tissues ($|logFC|>1$ and $P<0.05$ indicated significant difference) and the association between core genes and survival prognosis in HCC patients (logrank $P<0.05$ was considered statistically significant).

1.4 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis

In R software, the "clusterProfiler" package was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, with $P<0.01$ considered significant. The "ggplot2" package was used to visualize the analysis results.

1.5 Molecular Docking

The three-dimensional protein structures of core targets were downloaded from the PDB database (https://www.rcsb.org/), and water molecules and small molecule ligands were removed using PyMOL software. The 3D structure of wogonin compound was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and energy-minimized using Chem3D software. Finally, molecular docking was performed using AutoDock Vina, and the docking results were visualized in PyMOL.

1.6 Cell Culture

Human hepatocellular carcinoma cell line HepG2 and human normal cell line LO2 (both from Zhong Qiao Xin Zhou) were cultured in complete medium consisting of MEM (with NEAA) medium (90%) and fetal bovine serum (FBS) (10%). Cells were maintained at 5% CO_2 and 37°C.

1.7 CCK-8 Assay for Cell Viability

Cells were seeded at 1$×10^{4}$ cells per well in 96-well culture plates and incubated overnight at 5% CO_2 and 37°C. After removing the medium, each well was washed with 100 L PBS twice. Complete medium containing wogonin at concentrations of 0, 18.5, 37.5, 75.0, 150.0, and 300.0 mol/L was added, and cells were treated for 24, 48, and 72 hours. After removing the medium, each well was washed twice with 100 L PBS. CCK-8 reagent (ProNorn) was dissolved in complete medium at 10%, and 100 L was added to each well. Cells were incubated at 5% CO_2 and 37°C for 2 hours. Absorbance (OD value) was measured at 450 nm using a microplate reader.

1.8 Plate Colony Formation Assay

Cells were seeded in plates and incubated overnight at 5% CO_2 and 37°C. After removing the medium, cells were washed twice with 2 mL PBS. Complete medium containing wogonin at concentrations of 0, 37.5, 75.0, and 150.0 mol/L was added. After 48 hours of incubation, the medium was removed, and cells were washed twice with 2 mL PBS. Fresh complete medium was added, with medium changes every 3 days for 14 days. After removing the medium, cells were washed twice with 2 mL PBS, fixed with paraformaldehyde for 25-30 minutes, stained with crystal violet, and photographed.

1.9 Scratch Assay

HepG2 cells were seeded at 5$×10^{5}$ cells per well in 6-well plates and cultured until complete confluence. A sterile 100 L pipette tip was used to create a scratch wound. After washing twice with 2 mL PBS, serum-free medium containing wogonin at concentrations of 0, 37.5, 75.0, and 150.0 mol/L was added. Scratches were photographed at 0 and 48 hours, and analyzed using ImageJ software. Cell migration rate was calculated as the ratio of wound closure distance to the initial distance.

1.10 Cell Apoptosis Assay

HepG2 cells were seeded at 2$×10^{5}$ cells per well in 24-well plates and treated with complete medium containing wogonin at concentrations of 0, 37.5, 75.0, and 150.0 mol/L for 24 hours. Cell apoptosis was detected using an Annexin V-FITC kit. Cells were photographed under a fluorescence microscope after adding Annexin V-FITC reagent, with stronger green fluorescence signals indicating more apoptotic cells.

1.11 Western Blotting

HepG2 cells were treated with complete medium containing wogonin at concentrations of 0, 37.5, 75.0, and 150.0 mol/L for 48 hours. Total protein was extracted using RIPA lysis buffer containing protease inhibitors (Beyotime),

and protein concentration was determined using a BCA protein assay kit (Beyotime). After separation by 10% SDS-PAGE, proteins were transferred to PVDF membranes. Membranes were blocked with 5% skim milk at room temperature for 2 hours, then incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with IgG-conjugated secondary antibodies at room temperature for 2 hours. Finally, protein bands were detected using ECL.

1.12 Statistical Methods

Data are presented as ($\bar{x}\pm s$) (all experiments were repeated three times). GraphPad Prism software was used for statistical analysis and graphing. One-way ANOVA was used for comparisons among multiple groups, and t-test was used for comparisons between two groups. $P<0.05$ was considered statistically significant.

Results

2.1 Prediction Results of ADME Properties and Toxicity of Wogonin

The analysis results from SWISS ADME and ProTox-II-Prediction of Toxicity of Chemicals online platforms (Figure 1 [Figure 1: see original paper]) showed: molecular formula: $C_{16}H_{12}O_5$, molecular weight (MW): 284.26, rotatable bonds: 2, hydrogen bond acceptors: 5, hydrogen bond donors: 2, lipophilicity (MLOGP): 0.77, with high gastrointestinal absorption. Hepatotoxicity, carcinogenicity, mutagenicity, immunotoxicity, and cytotoxicity were all predicted as inactive.

2.2 Wogonin and Hepatocellular Carcinoma Targets

A total of 135 wogonin targets were collected from the TCMSP database, and 8,238 HCC targets were collected from the TTD, GeneCards, OMIM, and DisGeNET databases. The intersection of compound and disease targets yielded 113 potential targets for wogonin intervention in HCC (Figure 2 [Figure 2: see original paper]).

2.3 Protein-Protein Interaction Network Construction and Core Target Screening

The intersection targets were imported into the STRING database, yielding a protein-protein interaction network with 92 nodes (hiding nodes with degree of 0) and 265 edges (Figure 3 [Figure 3: see original paper]). Using the "MCC" algorithm in the "Cytohubba" plugin of Cytoscape software, the TOP 10 core targets were identified as: proto-oncogene tyrosine-protein kinase Src (SRC), heat shock protein HSP 90-alpha (HSP90AA1), cellular tumor antigen p53 (TP53), cyclin-dependent kinase 1 (CDK1), serine/threonine-protein kinase (AKT1),

G1/S-specific cyclin-D1 (CCND1), transcription factor Jun (JUN), RELA proto-oncogene (RELA), nitric oxide synthase (NOS2), and cyclin-dependent kinase inhibitor 1 (CDKN1A) (Figure 4 [Figure 4: see original paper]). Analysis of core gene mRNA expression levels in HCC patients and normal liver tissues based on TCGA data in the GEPIA database (Figure 5 [Figure 5: see original paper]) revealed that CDK1 and SRC expression levels were higher in HCC tissues than in normal liver tissues ($P<0.05$). High expression of CDK1, HSP90AA1, RELA, SRC, and JUN in HCC patients was associated with poor prognosis (Figure 6 [Figure 6: see original paper]) ($P<0.05$).

2.4 GO and KEGG Enrichment Analysis

GO enrichment analysis (Figure 7 [Figure 7: see original paper]A) showed that intersection genes were mainly enriched in biological processes (BP) such as response to oxidative stress, cellular response to chemical stress, and response to radiation; enriched in cellular components (CC) such as membrane raft and membrane microdomain; and enriched in molecular functions such as protein serine/threonine kinase activity, transmembrane receptor protein tyrosine kinase activity, and bile acid binding. KEGG pathway enrichment analysis (Figure 7B) revealed that intersection genes were mainly enriched in pathways including PI3K-Akt signaling pathway, p53 signaling pathway, and IL-17 signaling pathway.

2.5 Molecular Docking

The three-dimensional protein structures of core targets CDK1 and SRC were docked with the three-dimensional structure of wogonin compound using AutoDock Vina. The results showed that the binding energies of CDK1 and SRC with wogonin were both below -7 kcal/mol, demonstrating strong binding configuration activity between the proteins and compound (Figure 8 [Figure 8: see original paper]).

2.6 Wogonin Inhibits Proliferation of Liver Cancer Cells

CCK-8 assay revealed that HepG2 cell activity was significantly different among groups at 24, 48, and 72 hours ($P<0.05$) (Table 1). At the same concentration and time points, HepG2 cell activity was lower than LO2 cell activity, with statistically significant differences ($P<0.05$) (Tables 2 –4). Plate colony formation assay results showed significant differences in colony numbers among HepG2 cell groups ($P<0.05$) (Figure 9 [Figure 9: see original paper], Table 5).

2.7 Wogonin Inhibits Migration and Induces Apoptosis of Liver Cancer Cells

Scratch assay results indicated significant differences in HepG2 cell migration rates among groups ($P<0.05$) (Figure 10 [Figure 10: see original paper], Table 6). After treatment with certain concentrations of wogonin, HepG2 cells

were observed under a fluorescence microscope using an Annexin V-FITC kit, showing that green fluorescence signals intensified with increasing wogonin concentrations, indicating increased apoptosis (Figure 11 [Figure 11: see original paper]).

2.8 Wogonin Downregulates Expression of Core Targets CDK1 and SRC and Attenuates PI3K/AKT Signaling

Western blot results demonstrated that wogonin could downregulate the expression levels of core targets CDK1 and SRC within a certain concentration range. PI3K and P-AKT protein expression were downregulated, while total AKT protein expression was unaffected, indicating that wogonin attenuated PI3K/AKT signaling pathway activity (Figure 12 [Figure 12: see original paper], Table 7).

Discussion

The ADME characteristics of wogonin were found to comply with Lipinski's Rule of Five for small molecule drug-likeness [14]. Toxicity prediction results showed no hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity, or immunotoxicity, indicating its potential for drug development. Analysis of the TOP10 core targets for wogonin intervention in HCC revealed that previous studies have shown AKT1 deletion can prevent tumor formation in mice [15], CCND1 silencing can inhibit differentiation of liver cancer stem cells [16-17], TP53 mutations leading to downregulated immune response are associated with HCC prognosis [18-20], and silencing CDKN1A can promote proliferation and migration of HCC [21-22]. CDK1 is highly expressed in HCC and associated with poor prognosis, and downregulating CDK1 expression can inhibit proliferation, migration, and induce apoptosis in HCC cells [23-25]. High SRC expression promotes HCC progression, and inhibiting SRC expression significantly suppresses proliferation of liver cancer cells [26-28]. In KEGG enrichment analysis, the IL-17 signaling pathway can promote HCC progression, and targeting this pathway can inhibit proliferation of HCC cells [29-30]. Targeting the p53 signaling pathway can regulate HCC development and progression [31-32]. The PI3K/AKT signaling pathway is associated with cell proliferation, migration, and apoptosis, is aberrantly activated in cancer, and is involved in tumor development and progression, making it an effective therapeutic target in cancer treatment [33-34]. Network pharmacology analysis identified that the mRNA expression levels of two core targets, CDK1 and SRC, were significantly higher in HCC tissues than in normal liver tissues, and high expression of CDK1 and SRC in HCC patients was associated with poor survival prognosis. The PI3K/AKT signaling pathway had the highest number of enriched genes.

In vitro experiments demonstrated that wogonin at certain concentrations could inhibit proliferation of human HCC cell line HepG2, while its inhibitory effect on proliferation of normal human liver cell line LO2 was less pronounced at the same concentrations, suggesting that wogonin exhibits specific inhibitory activity against liver cancer cells. Scratch assay and apoptosis experiments showed

that wogonin could inhibit migration of liver cancer cells and induce apoptosis. Western blot results revealed that after wogonin treatment, expression of core targets CDK1 and SRC decreased, and PI3K/AKT signaling pathway activity was attenuated.

Based on in vitro experiments and previous literature, wogonin may interfere with HCC development and progression by inhibiting expression of core genes CDK1 and SRC, attenuating PI3K/AKT pathway signaling, thereby suppressing proliferation and migration of liver cancer cells and inducing apoptosis. Combined with network pharmacology analysis and in vitro experimental results, this study demonstrates that wogonin is a natural compound with potential for development as a therapeutic agent for HCC. This research provides a preliminary exploration of the mechanism of wogonin in treating HCC and offers a reference for its future development and utilization.

Limitations: This study only performed in vitro experimental validation on core genes that showed significant differences in mRNA expression levels between HCC tissues and normal liver tissues and were associated with poor survival prognosis, without conducting in-depth analysis of other core genes. Due to experimental constraints, further in vitro studies on the intervention effects of wogonin on HCC development and progression could not be performed.

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Author Contributions

Yang Yongfeng conceived the research idea and designated the overall research objectives. Yang Anyin and Liu Hongli performed data analysis, experimental operations, and manuscript writing. Chen Miaoyang, Zheng Yufeng, and Xu Zhiyuan collected relevant literature and materials. This article has no conflicts of interest.

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