

Bioinformatic Analysis and Computer-Aided Study to Investigate the Potential Application of a Bioflavonoid Compound Bilobetin in Liver Cancer Treatment

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Abstract

Background: Hepatocellular carcinoma (HCC/LIHC) is the most common type of primary liver cancer, which is a leading cause of cancer death worldwide. Patients with HCC have a short survival time after diagnosis. Unfortunately, there are no effective treatments for advanced or aggressive HCC. Thus, the rapid development of new therapeutic drugs or treatment methods for HCC is urgently needed.

Methods: Bioinformatic tools and computer-aided predictions advance the processes of drug development. In this study, we incorporated bioinformatic analyses and computer-aided drug development processes to investigate the potential application of bilobetin, a bioactive compound of bioflavonoid, as a therapeutic agent for HCC treatment. **Results:** Our results revealed that 4 out of 20 predicted hub target genes of bilobetin displayed functional importance in cancer-related signaling pathways in different cancers, including HCC. Importantly, the mRNA expression levels of these four key hub genes (*VEGFA*, *SRC*, *MMP9*, and *CDKI*) were significantly different between normal and HCC tumor samples. Their expression levels were significantly associated with the clinical survival outcomes of HCC patients, as well as the immune cell infiltration levels in the HCC tumor microenvironment. In addition, these four genes showed significant co-expression correlated with immune checkpoint genes, including *CD274*, *PDCD1*, *CTLA4*, and *CD47*. Furthermore, we used computer-aided approaches to investigate the binding affinity and potential binding mechanisms between bilobetin and target proteins encoded by four key hub genes. **Conclusions:** In conclusion, our study shed light on the potential application of the bioactive bioflavonoid molecule bilobetin in LIHC treatment by regulating four key hub genes.

Keywords: primary liver cancer; hepatocellular carcinoma; bioactive compound; bilobetin; bioinformatics; molecular docking; protein-ligand interaction; treatment

1. Introduction

The most prevalent type of primary liver cancer is hepatocellular carcinoma (HCC/LIHC), one of the leading causes of cancer death worldwide [1,2]. The number of HCC cases is increasing [3]. It has been estimated that there will be more than 1 million cases per year globally by 2025 [4]. HCC can be induced by many factors, such as extensive alcohol consumption, non-alcoholic fatty liver disease (NAFLD), liver fibrosis or cirrhosis. The incidence of NAFLD-associated HCC is increasing, which is also commonly associated with many other metabolic diseases such as obesity, type 2 diabetes mellitus, and cardiovascular disease [5–7]. Most HCC cases are diagnosed at a late stage; however, current therapies for advanced HCC are not promising [8,9]. Therefore, the exploration of early diagnostic biomarkers and development of effective treatment drugs or strategies for HCC are urgently needed [10].

Research studies and clinical trials have demonstrated that *Ginkgo biloba* extract displays effective activities in

the treatment of metabolic diseases, including cancers (e.g., brain and breast cancers) [11]. Bilobetin is a bioactive molecule isolated from the leaves of the *Ginkgo biloba*. As the bioflavonoid class of phytochemicals, bilobetin exhibits the pharmacological characteristics of *Ginkgo biloba* extract [12]. The important pharmacological properties of bilobetin include antioxidant, anti-inflammatory, anti-hyperlipidemic, and anti-proliferative activities [12–14]. Studies have also revealed that bilobetin possesses potent anti-cancer activity [11,14,15]. For example, bilobetin displays cytotoxic effects against several human cancer cell lines [14], including cervical carcinoma (HeLa), large cell lung cancer (NCI-H460), lymphoma (Daudi), myelogenous leukemia (K562), ovarian adenocarcinoma (SKOV3), pancreatic carcinoma (MIAPaca-2), and breast carcinoma (MCF-7). However, the action of bilobetin in liver cancer treatment remain to be studied.

In this study, we investigated the activity of bilobetin in liver cancer treatment. First, we applied a computer-aided drug design tool, SwissTargetPrediction, to predict

molecular targets of bilobetin based on the data generated from both laboratory experiments (binding assay) and the calculation of similarities between 2D/3D structures of query molecules and known molecules from online databases [16,17]. Then, we analyzed protein-protein interaction (PPI), KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, and gene ontology (GO) pathway to examine the functions of target genes, including biological processes (BP), cellular components (CC), and molecular functions (MF). Among the target genes, we first identified 20 hub genes using Cytoscape, and then further revealed the functions and enriched signaling pathways of the hub genes based on the analyses of KEGG and GO pathways [18,19]. We subsequently applied bioinformatic tools, such as UALCAN (the University of Alabama at Birmingham CANcer Data Analysis Portal) [20,21], to analyze the expression levels of hub genes in clinical LIHC/HCC tumor and non-tumor control samples from the Cancer Genome Atlas (TCGA) database. Meanwhile, the association of hub genes with the prognostic outcomes of LIHC patients was also examined [22,23]. Interestingly, we found that four key genes out of 20 hub genes showed significantly different mRNA expression levels between clinical tumor and non-tumor samples, and their mRNA expression levels were significantly associated with the clinical prognostic outcomes (survival rates) of LIHC patients. In addition, we analyzed the proteomic expression levels of these four key genes between normal and primary LIHC tumor samples [24,25].

Furthermore, we evaluate the correlation between the expression levels of four key genes with levels of immune cell infiltration [26,27] and the expression of immune checkpoints such as *CD274*, *PDCD1*, *CTLA4*, and *CD47* in the HCC tumor microenvironment [28,29]. Finally, computer-aided programming was used to explore the underlying molecular mechanism of bilobetin as a therapeutic agent for LIHC therapy by regulating four key genes. The binding affinities and modes of bilobetin with four target proteins were tested using computer-based molecular docking methods [30]. The key amino acids that were involved in the interactions between the bilobetin molecule and proteins encoded by four key genes were analyzed, including the hydrophobic interaction and formation of hydrogen bonds [31,32].

2. Materials and Methods

2.1 Drug Target Prediction

SwissTargetPrediction is a commonly used bioinformatic tool to predict the targets of bioactive small molecules (e.g., bilobetin) in humans and other vertebrates. It is a useful approach for understanding molecular mechanisms, rationalizing possible side-effects, and predicting off-targets of known molecules [14]. In this study, it was used to investigate the molecular targets of bilobetin by selecting *Homo sapiens* genes.

2.2 Construction of the Protein-Protein Interaction Network

We applied a bioinformatic database, STRING (<https://string-db.org/>), for the construction of a protein-protein interaction (PPI) network. This tool allows the construction of an interaction network based on the data obtained from both experiments and online databases. The cross-interaction of multiple queried proteins can be predicted using this tool [33]. For parameters used in this study, *Homo sapiens* genes were selected, and 0.4 was set as the default threshold, which was considered significant for the minimum required interaction specificity score.

2.3 GO Functional Enrichment and KEGG Signaling Pathway Enrichment Analyses

The Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to investigate the functional enrichment and signaling pathway enrichment of bilobetin-targeted genes and 20 hub genes. The categories of the GO enrichment analysis included biological processes (BP), cellular components (CC), and molecular functions (MF). We applied DAVID bioinformatics resources for the analysis of results and used the platform (<http://www.bioinformatics.com.cn>) for result plotting and visualization [34,35].

2.4 Cytoscape

The Cytoscape (<https://cytoscape.org/>) platform was applied for the analysis and visualization of the complex networks including the connecting nodes and molecular interactions, as well as the identification of the key hub genes from bilobetin-targeted genes [36].

2.5 Gene Expression Profiling Interactive Analysis (GEPIA)

The interactive bioinformatics tool GEPIA (<http://gepia.a.cancer-pku.cn/>) [37] was applied to compare the gene expression levels of target genes between normal samples and LIHC tumor samples and to analyze the survival outcomes of LIHC patients. In this study, to compare the expression levels of target genes in the normal and tumor samples from the database, the matched normal data included the TCGA normal data and GTEx data. ANOVA method was applied with the default parameters ($\log_2FC = 1$; $p\text{-value} = 0.01$). The analysis of the survival outcomes of LIHC patients was calculated using the overall survival times, and the hazard ratio was calculated based on the Cox PH Model. A p -value less than 0.05 was defined as statistically significant.

2.6 Tumor Immune Estimation Resource (TIMER)

TIMER is a resource with multiple modules allowing for a comprehensive and systematic examination of immune cell infiltration levels in clinical samples for different cancer types [38–40]. In this study, we applied the gene module to investigate the correlation between the expression levels of identified key hub genes and the immune

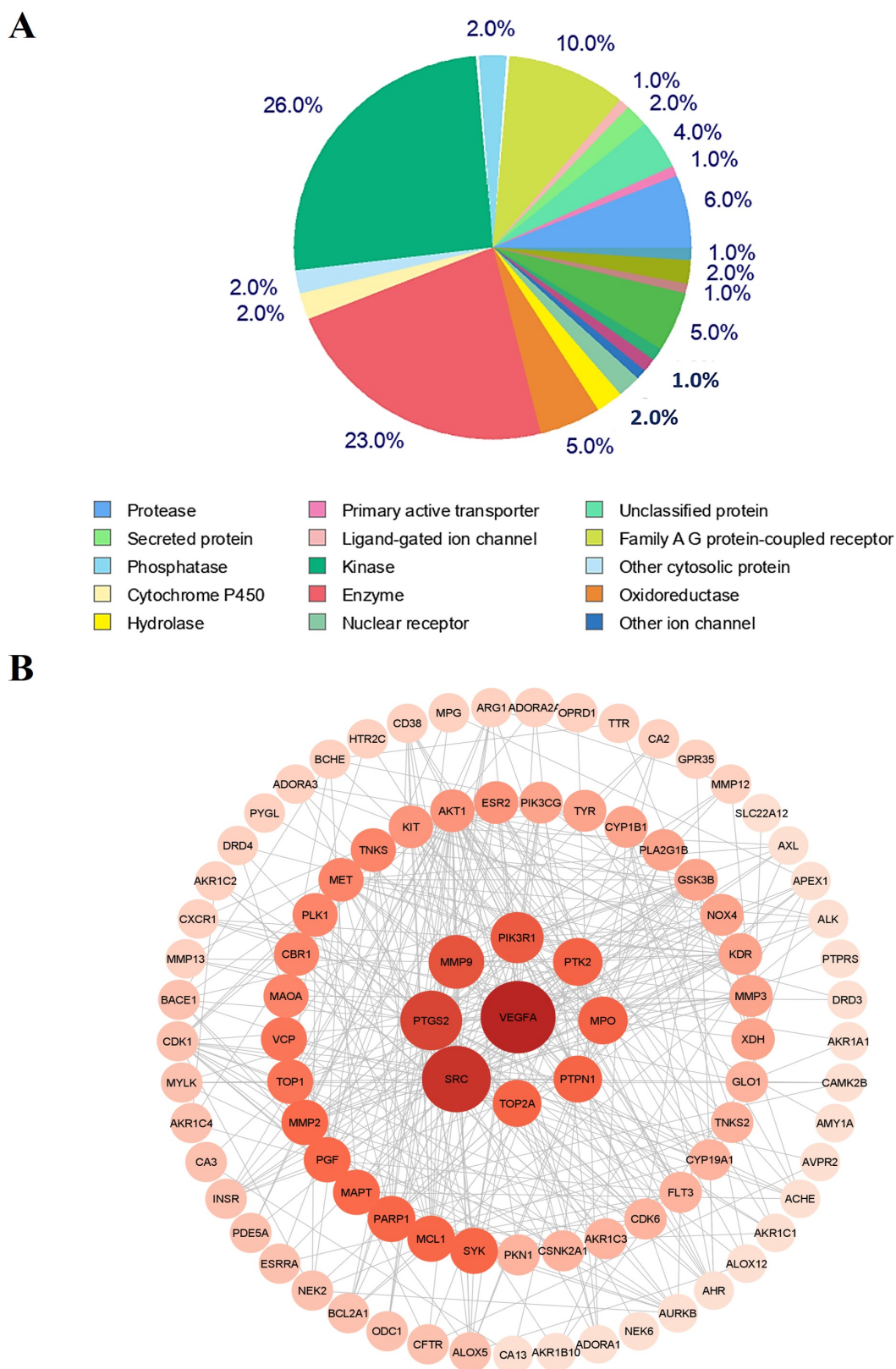


Fig. 1. Predicted drug targets of bilobetin and the protein-protein interaction (PPI) network of targets. (A) The pie chart shows the bioactivity categories of bilobetin-targeted proteins and the percentages of each category. (B) The constructed PPI network of all target proteins of bilobetin.

GO analysis of targeted genes

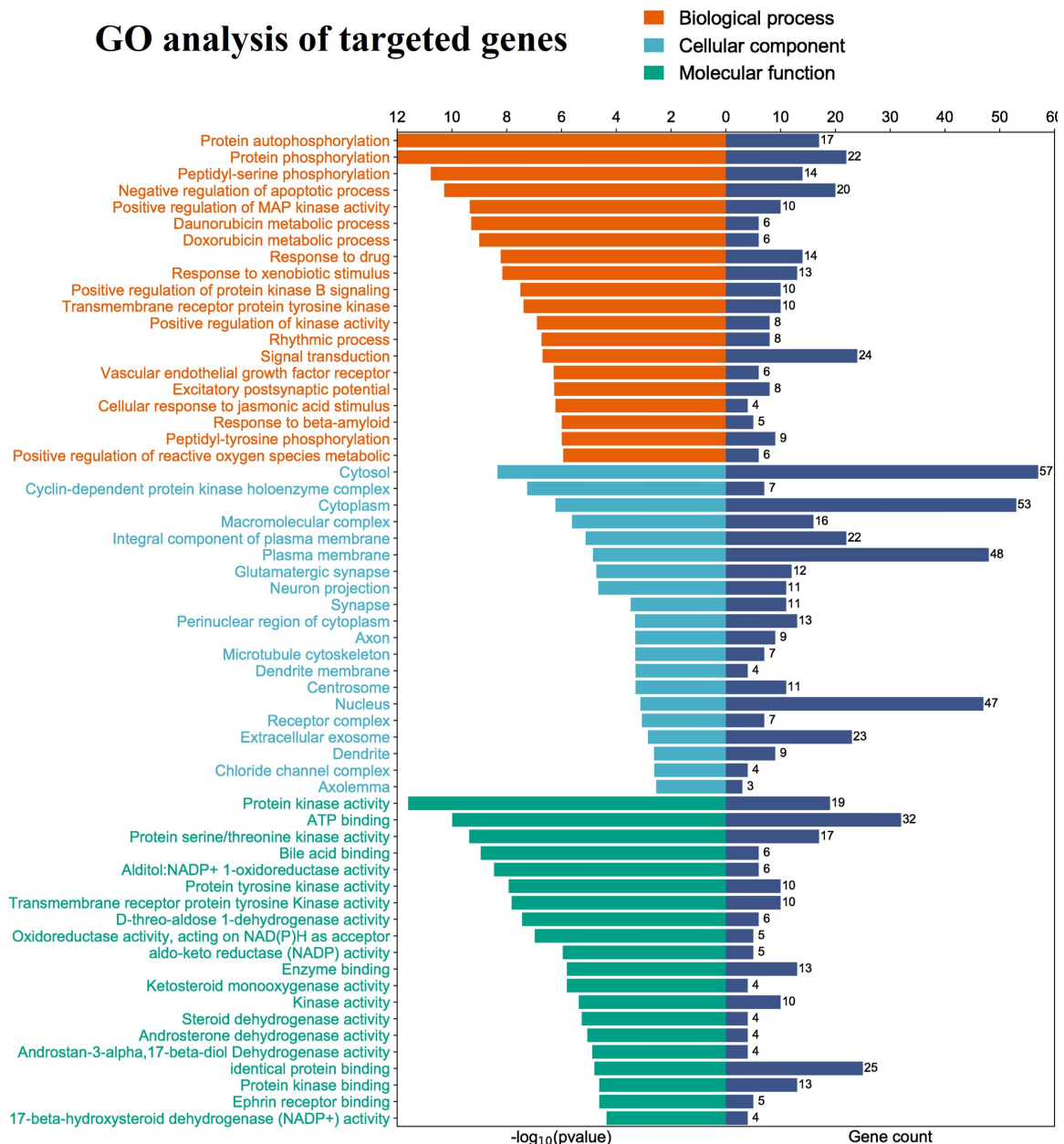


Fig. 2. The gene ontology (GO) enrichment analysis results. GO analysis results display the roles of bilobetin-targeted genes in biological processes (orange), molecular function (green), and cellular components (blue).

cell infiltration levels in LIHC. The correlation module was applied to analyze and generate the scatterplots of the co-expression levels between the identified key genes and the immune checkpoint-associated genes in LIHC. The scatterplots were generated and presented based on the purity-corrected partial Spearman's Rho value, and $p < 0.05$ was defined as statistical significance.

2.7 Molecular Docking and Interaction Analysis of the Protein-Ligand Complex

Molecular docking was performed using Cavity-detection guided Blind Docking (CB-DOCK), the open-source program AutoDock Vina v.1.2.0. (<http://www.vina.scripps.edu/>) [41], for testing the binding affinity and simulation of the binding mechanism. The crystal structures of proteins encoded by the identified genes used for docking were obtained from the PDB database, which were well-prepared for the docking program, including VEGFA (PDB ID: 5T89); SRC (PDB ID: 2H8H); MMP9 (PDB ID: 4H2E); CDK1 (PDB ID: 5HQ0). Pymol software was used for the visualization and presentation of protein-ligand interaction. PDBsum [42] and Discovery Studio Visualizer v21.1.0.20298 (BIOVIA Dassault Systemes, San Diego, CA, USA) were used for the 2D interaction analysis and presentation.

Table 1. The results of molecular docking between bilobetin and gene-coded proteins.

Gene	PDB ID	Cavities	Volume	Center_X	Center_Y	Center_Z	Size_X	Size_Y	Size_Z	Score
<i>VEGFA</i>	5T89	5	54	-62.038	32.762	55.268	24	24	24	-8.8
<i>SRC</i>	2H8H	4	697	17.699	25.222	57.424	24	24	24	-10.6
<i>MMP9</i>	4H2E	2	563	-5.979	0.783	21.686	24	24	24	-11.0
<i>CDK1</i>	5HQ0	1	1438	28.971	-65.752	187.146	24	24	24	-10.0

2.8 Pharmacokinetics and Drug-Likeness

The pharmacokinetics, drug-likeness, and medicinal chemistry of bilobetin were evaluated using the SwissADME bioinformatics tool [43].

2.9 The mRNA Expression Levels of Immune Checkpoints

The mRNA expression levels of immune checkpoint genes *PDCD1*, *CD274*, *CD47*, and *CTLA4* were analyzed using UALCAN web-based tool. The required gene expression level in LIHC was analyzed to compare the normal and primary tumor groups using Student's *t*-test, and $p < 0.05$ was considered statistically significant [20,21].

2.10 Immunohistochemical Staining (IHC)

The Human Protein Atlas (<https://www.proteinatlas.org/>, version 23.0) [44,45] was applied for the generation of a representative IHC-staining panel to demonstrate the pathologic effects associated with different protein expression levels of key genes *VEGFA*, *SRC*, *MMP9*, and *CDK1*. This database was also used to validate the association between the expression levels (low and high) of four key genes and the survival outcomes of LIHC patients.

2.11 Statistical Analysis

Student's *t*-test (considering unequal variance) was used to compare mRNA expression levels of key genes in normal samples and LIHC tumor samples, and $p < 0.05$ was defined as statistical significant between groups. Kaplan Meier-plotter was adopted for analyzing and generating the survival curve. A log-rank test was applied to measure the significance of survival impact. A log-rank $p < 0.05$ was defined as the cut-off value for statistical significance.

3. Results

3.1 Drug Targets of Bilobetin in Prediction and Protein-Protein Network Construction of Target Genes

To investigate the drug targets of bilobetin, we applied one of the computer-aided drug discovery tools, SwissTargetPrediction, which can predict drug targets of a small molecule based on the datasets generated from well-designed experimental binding assays and the online database. *Homo sapiens* was defined as a species for the identification of target proteins. The obtained results showed that among the predicted targets, 26% were kinases, 23% were enzymes, 10% belonged to family A of G protein-coupled receptors, 6% were proteases, whereas 5% were oxidoreductases and 5% were lyases (Fig. 1A). Other

targets include cytochrome P450, hydrolases, isomerases, nuclear receptors, cytosolic proteins, phosphatases, and secreted proteins (2% for each class category), electrochemical transporters, erasers, ligand-gated ion channel proteins, other ion channel proteins, phosphodiesterases, primary active transporters, and transcription factors (1% for each class category) (Fig. 1A).

To further examine the properties of the bilobetin-targeted proteins and their interaction partners, we applied the bioinformatic analysis tool, STRING, to construct a PPI network of these targets (Fig. 1B). The constructed network contained 98 nodes and 386 edges, with an average node degree of 7.88 and an average local clustering coefficient value of 0.54. The PPI enrichment *p*-value was less than 1.0×10^{-16} . These results showed that the input query proteins had a significant interaction in the constructed network among themselves, rather than from the random connection based on the same size and degree distribution of proteins in the database. The PPI analysis results have demonstrated the input query targets belong to a biologically connected group among themselves. Based on this result, we further performed the subsequent analysis by focusing on the biological functions and related signaling pathways of the targets.

3.2 KEGG Pathway Analysis and GO Enrichment Analysis of Target Genes

To determine the biological and functional enrichment of target genes of bilobetin, we performed GO enrichment analysis. The GO enrichment analysis results for biological processes revealed that the target genes play important roles in protein autophosphorylation, apoptotic process, mitogen-activated protein (MAP) kinase activity, metabolic processes of chemotherapy drugs such as doxorubicin and daunorubicin, vascular endothelial growth factor receptor, and metabolic regulation of reactive oxygen species (Fig. 2). For the cellular component, the results showed these genes were mostly involved in the cytosol, cyclin-dependent protein kinase holoenzyme complex, cytoplasm, plasma membrane, glutamatergic synapse, neuron projection, perinuclear region of cytoplasm, axon, and others (Fig. 2). The results of the molecular function analysis identified that the key roles of these genes were enriched for protein kinase activity, protein serine/threonine kinase activity, ATP binding, bile acid binding, oxidoreductase activity, tyrosine kinase activity, and others (Fig. 2).

To better understand the major roles of these targeted genes in signaling pathways and diseases, we further per-

KEGG analysis of targeted genes

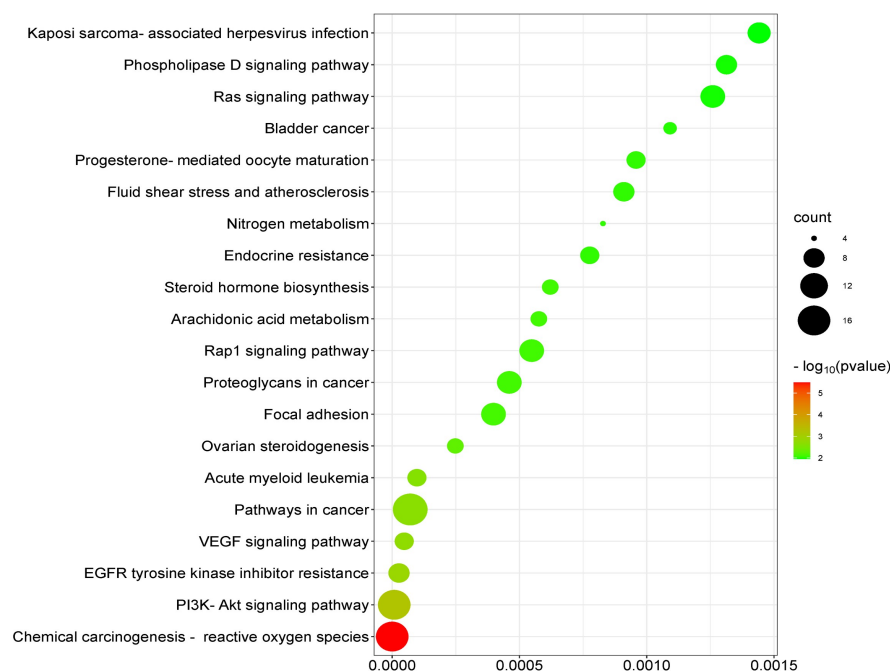


Fig. 3. The Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis of bilobetin-targeted genes. The top 20 signaling pathways, functional information, and related diseases are presented in the bubble plot. The size of the bubbles represents the gene count, and the color of the bubbles represents the values of $-\log_{10}(p\text{-value})$.

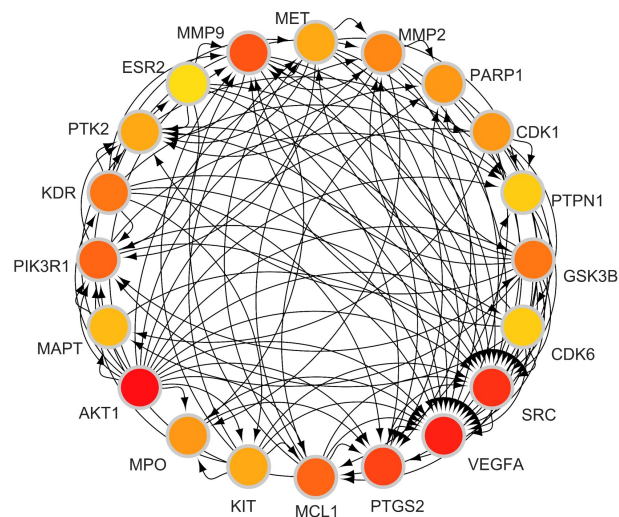


Fig. 4. Identification of the top 20 hub genes using Cytoscape based on the results of protein-protein interaction (PPI) network of bilobetin-targeted genes. The PPI network contains 20 nodes and 113 edges. The average node degree is 11.3, and the average local clustering coefficient is 0.735. The PPI enrichment p -value is less than 6.1×10^{-6} , indicating there is a significant interaction among the input proteins.

formed a KEGG analysis and the top 20 enriched signaling pathways are shown (Fig. 3). The results showed that these genes played critical roles in chemical carcinogenesis-reactive oxygen species, pathways in cancer, acute myeloid leukemia, bladder cancer, and proteoglycan in cancer. The enriched signaling pathways of target genes were the RAS signaling pathway, PI3K-AKT signaling pathway, VEGF signaling pathway, and EGFR tyrosine kinase inhibitor resistance.

3.3 Identification of the Hub Genes from Bilobetin-Targeted Genes

To further identify linkage genes from bilobetin-targeted genes, we analyzed hub genes based on the previously constructed protein-protein interaction (PPI) results using the software Cytoscape. The top 20 hub genes were *MET*, *MMP2*, *PARP1*, *CDK1*, *PTPN1*, *GSK3B*, *CDK6*, *SRC*, *VEGFA*, *PTGS2*, *MCL1*, *KIT*, *MPO*, *AKT1*, *MAPT*, *PIK3R1*, *KDR*, *PTK2*, *ESR2*, and *MMP9*, and were identified to have a complex interaction (Fig. 4).

3.4 Validation of the Functional Enrichment and Signaling Pathways of Hub Genes

To further test whether the top 20 hub genes represent the main functions of target genes, the examination of functional enrichment and signaling pathways of these hub genes was carried out. The results of GO functional enrichment analysis revealed that, for biological processes,

GO analysis of 20 hub genes

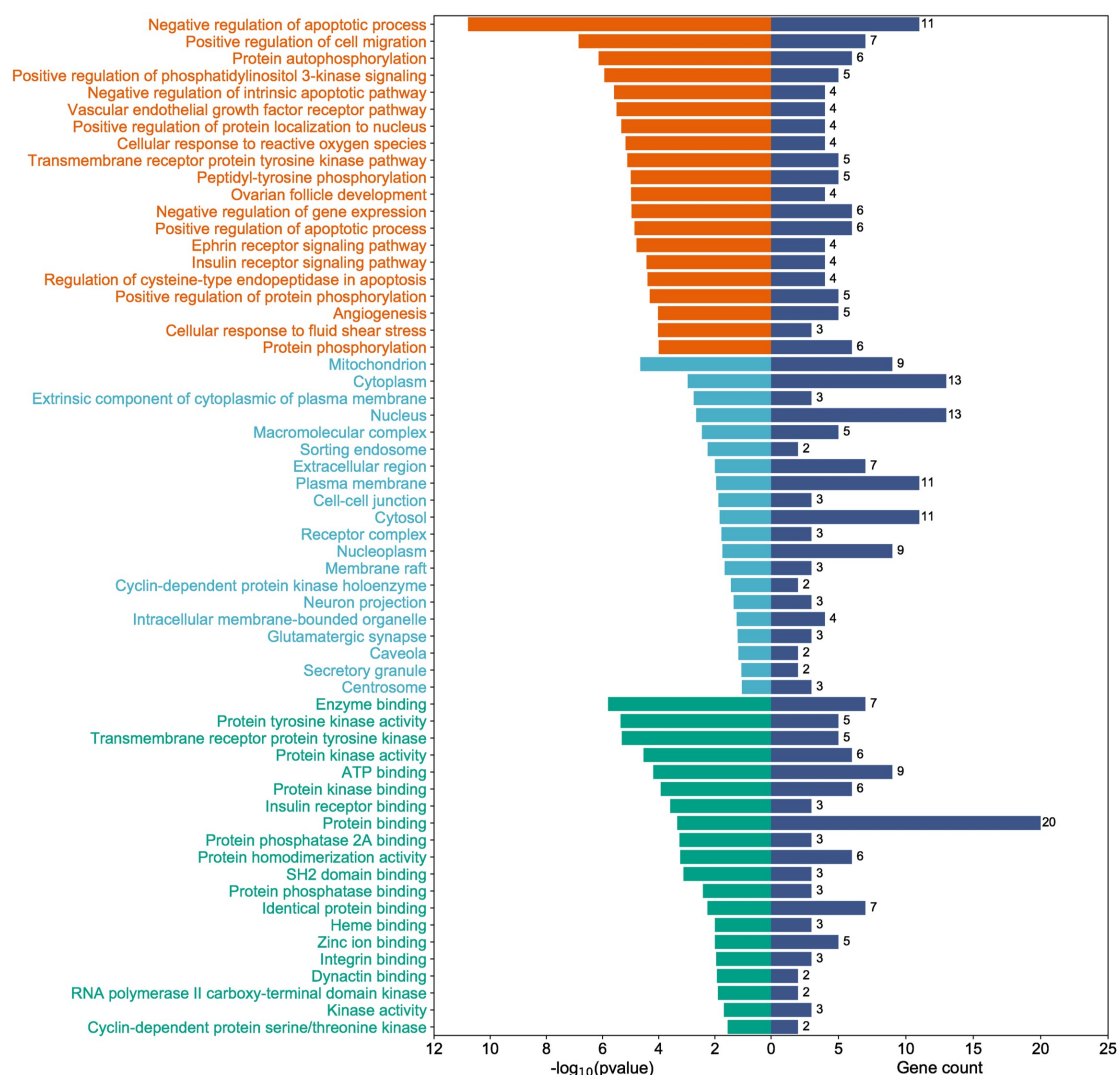


Fig. 5. The gene ontology (GO) enrichment analysis results of 20 hub genes. The top 20 functionality enrichment items for each category were presented, including biological processes (orange), molecular functions (green), and cellular components (blue).

20 hub genes were highly enriched in the following pathways including negative regulation of the apoptotic process, positive regulation of cell migration, protein autophosphorylation, positive regulation of phosphatidylinositol 3-kinase signaling pathway, negative regulation of the intrinsic apoptotic pathway, vascular endothelial growth factor receptor pathway, positive regulation of protein localization to nucleus, cellular response to reactive oxygen species, transmembrane receptor protein tyrosine kinase pathway, and peptidyl-tyrosine phosphorylation (Fig. 5). Regarding the cellular component, these hub genes were found to have greater enrichment in the mitochondrion, cytoplasm, the extrinsic component of cytoplasmic membrane, nucleus, macromolecular complex, sorting endosome, extracellular region, plasma membrane, cell-cell junction, cytosol, *etc.*

(Fig. 5). The results of the molecular function demonstrated that the hub genes were highly enriched in the function of enzyme binding, protein tyrosine kinase activity, transmembrane receptor protein tyrosine kinase, protein kinase activity, ATP binding, protein kinase binding, insulin receptor binding, protein binding, protein phosphatase 2A binding, protein homodimerization activity, and other molecular function processes (Fig. 5).

Most importantly, the subsequent analysis of KEGG signaling pathways uncovered that 20 hub genes were highly enriched in and contributed to the important signaling pathways in cancer biology, such as the VEGF signaling pathway, pathways and proteoglycans in cancer, microRNAs in cancer, EGFR tyrosine kinase inhibitor resistance, PI3K-AKT signaling pathway, chemical carcinogenesis-

KEGG analysis of 20 hub genes

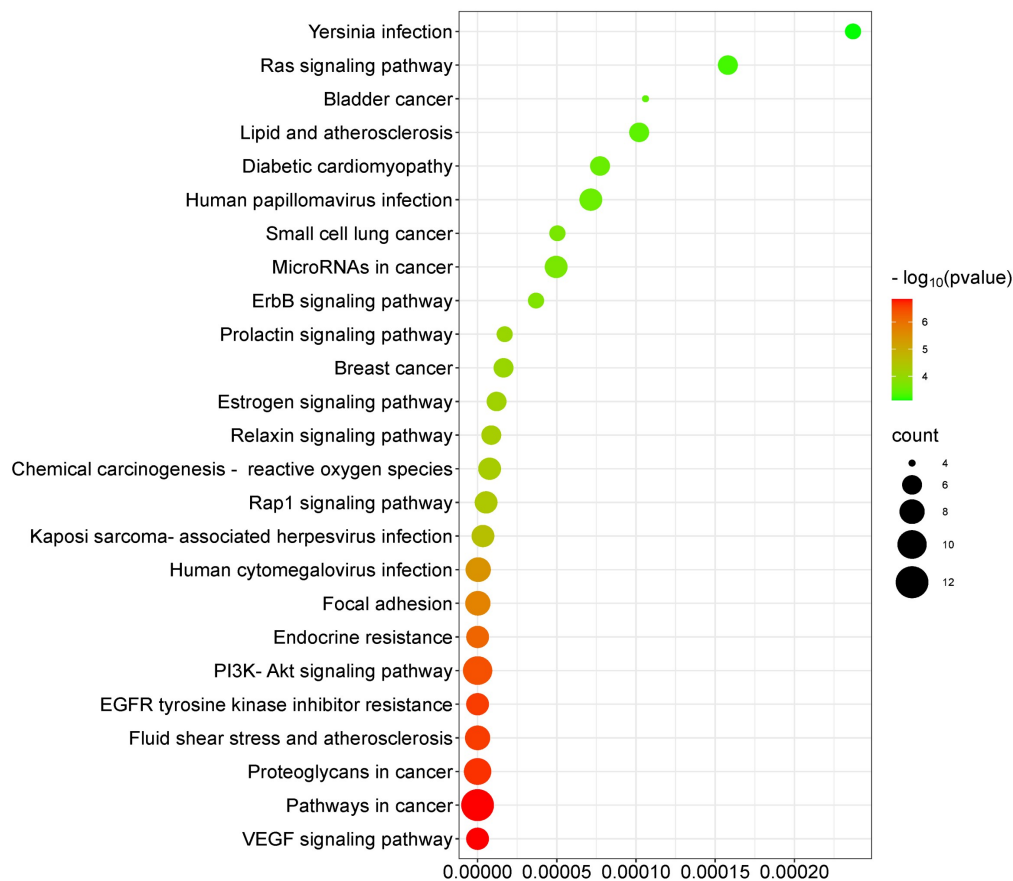


Fig. 6. The Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis of 20 hub genes. The top 25 signaling pathways, associated diseases, and other functional information were presented in the bubble plot. The size of the bubbles represents gene counts, and the color of the bubbles represents the value of $-\log_{10}(p\text{-value})$.

reactive oxygen species, ErbB signaling pathway, small cell lung cancer, breast cancer, bladder cancer, and RAS signaling pathway. The top 25 signaling pathways and related diseases are shown (Fig. 6).

3.5 Examining the mRNA Expression Levels of Hub Genes in LIHC Tumor and Control Samples

Due to the important roles of bilobetin-targeted hub genes in cancer, we extended our study by examining the mRNA expression levels of these hub genes in normal and liver tumor samples using a clinical database. Among the 20 hub genes, we identified 6 genes that showed significant differences in mRNA expression levels between the normal/non-tumor ($n = 160$) and LIHC tumor samples ($n = 369$). The expression level of *VEGFA* was significantly decreased in LIHC tumor samples compared with normal samples, whereas the expression levels of *SRC*, *MMP9*, *PARP1*, *CDK1*, and *PTK2* were significantly increased in LIHC tumor samples compared with the normal samples (Fig. 7).

3.6 Exploring the Association between the Clinical Prognosis Outcomes of LIHC Patients and the Expression Levels of Hub Genes

To further explore the clinical prognosis values of hub genes, we examined the association between the clinical outcomes of LIHC patients and the expression levels of 20 hub genes using the bioinformatics tool GEPIA. The results showed that, among 20 hub genes, the expression levels of eight genes (*VEGFA*, *SRC*, *MMP9*, *GSK3B*, *CDK1*, *KIT*, *MAPT*, *PTPN1*) were significantly associated with the clinical survival outcomes of LIHC patients. The expression levels of these eight genes were significantly and negatively associated with the survival outcomes of LIHC patients (Fig. 8).

Given the mRNA expression levels of four hub genes (*VEGFA*, *SRC*, *MMP9*, and *CDK1*) and their association with the survival outcomes of LIHC patients, we then focused on these four key hub genes in the following study.

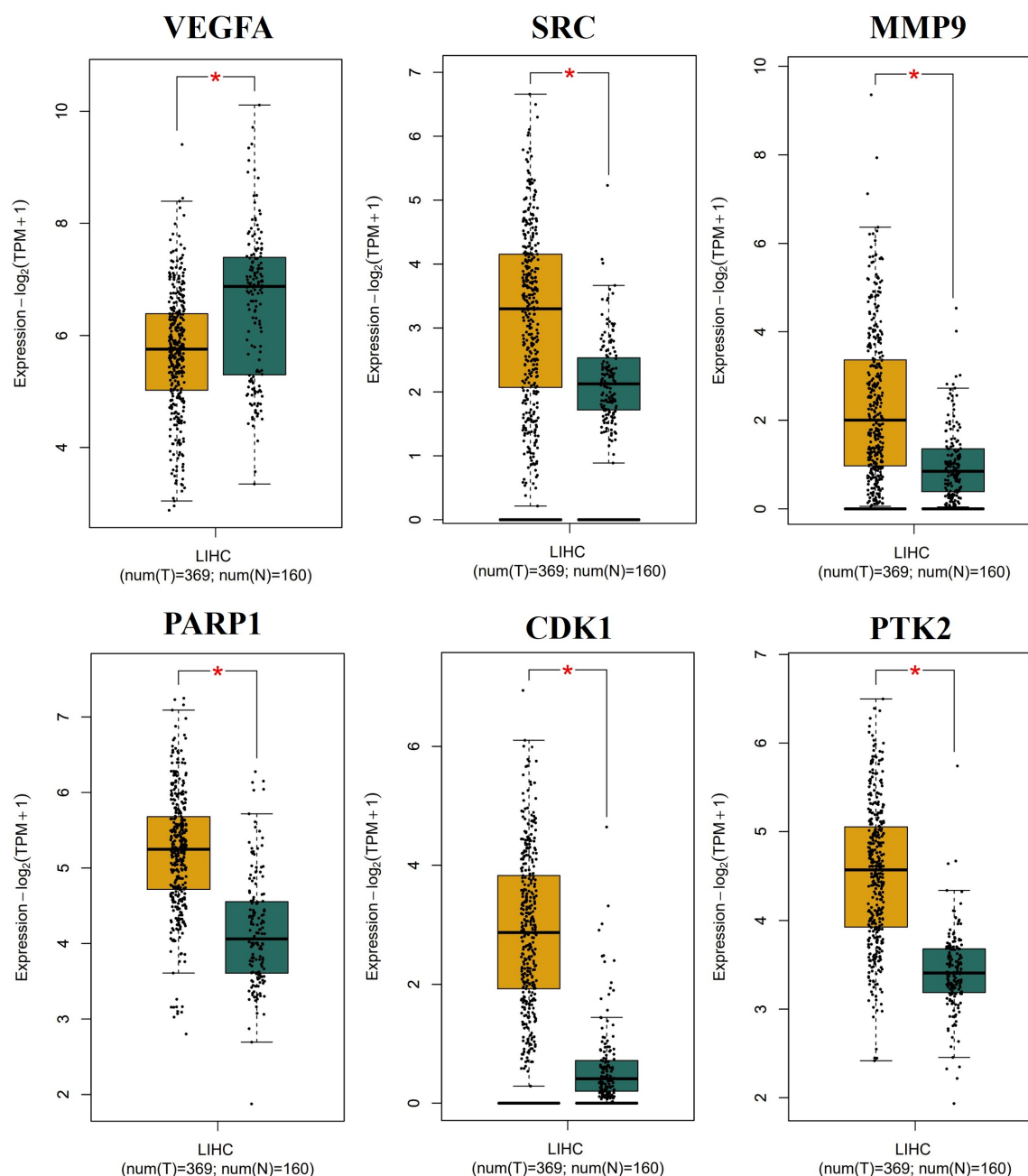


Fig. 7. Analysis of mRNA expression levels of hub genes between non-tumor control and liver hepatocellular carcinoma (LIHC) tumor samples. Among the 20 hub genes, *VEGFA*, *SRC*, *MMP9*, *PARP1*, *CDK1*, and *PTK2* showed significantly different expression levels between non-tumor and tumor samples. $n = 160$ (non-tumor samples) and $n = 369$ (tumor samples). ANOVA was applied for statistical analysis, and $*p < 0.05$ was defined as statistically significant.

3.7 Examination of the Proteomic Expression Levels of Four Key Hub Genes in Normal Liver Tissues and Primary LIHC Tumor Samples

To analyze the proteomic expression levels of four genes, including *VEGFA*, *SRC*, *MMP9*, and *CDK1*, we used the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database to compare their proteomic expression levels between normal samples and primary LIHC tumor samples. Our results showed that the proteomic expression levels of *VEGFA*, *MMP9*, and *CDK1* have significantly dif-

ferent. However, there was no statistically significant difference in the proteomic expression levels of *SRC* between normal and LIHC tumor samples (Fig. 9).

To validate the significance of the four key hub genes (*VEGFA*, *SRC*, *MMP9*, and *CDK1*) in LIHC, the protein expression staining results and the prognosis values of the four key genes in the overall survival of HCC patients were collected from the Human Protein Atlas for analysis. The prognosis values were consistent with the results

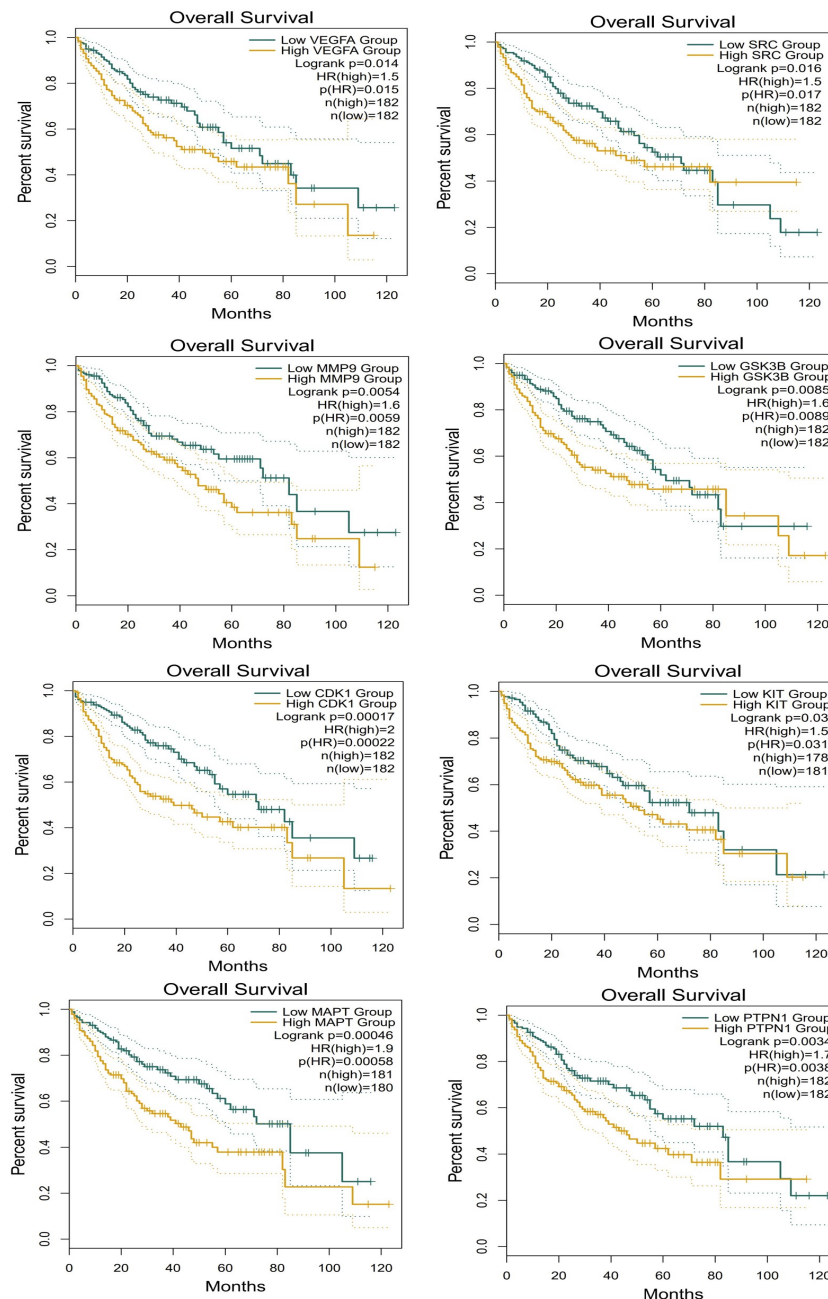


Fig. 8. Association of the mRNA expression levels of 20 hub genes with the overall survival outcomes of LIHC patients analyzed by comparing high and low gene expression groups. Among 20 hub genes, the gene expression levels of *VEGFA*, *SRC*, *MMP9*, *GSK3B*, *CDK1*, *KIT*, *MAPT*, and *PTPN1* were significantly associated with the clinical outcomes in terms of overall survival times (months) of LIHC patients. The hazard ratio was calculated based on the Cox PH Model according to the default parameters, as well as the 95% confidence interval (CI).

shown in Fig. 8, indicating that high expression of these four genes predicted poor overall survival of HCC patients (Supplementary Fig. 1). In addition, immunohistochemical (IHC) staining showed low and high expression levels of the proteins encoded by the four key genes in clinical LIHC samples (Supplementary Fig. 1).

3.8 Evaluation of the Correlation of Immune Cell Infiltration with the Expression of Four Key Genes

Due to the critical roles of tumor-infiltrating immune cells in the survival outcomes of LIHC patients, it is important to examine the correlation of the expression levels of the four key hub genes (*VEGFA*, *SRC*, *MMP9*, and *CDK1*) with the tumor-infiltrating levels of immune cells, as well as the expression of immune checkpoint-associated genes.

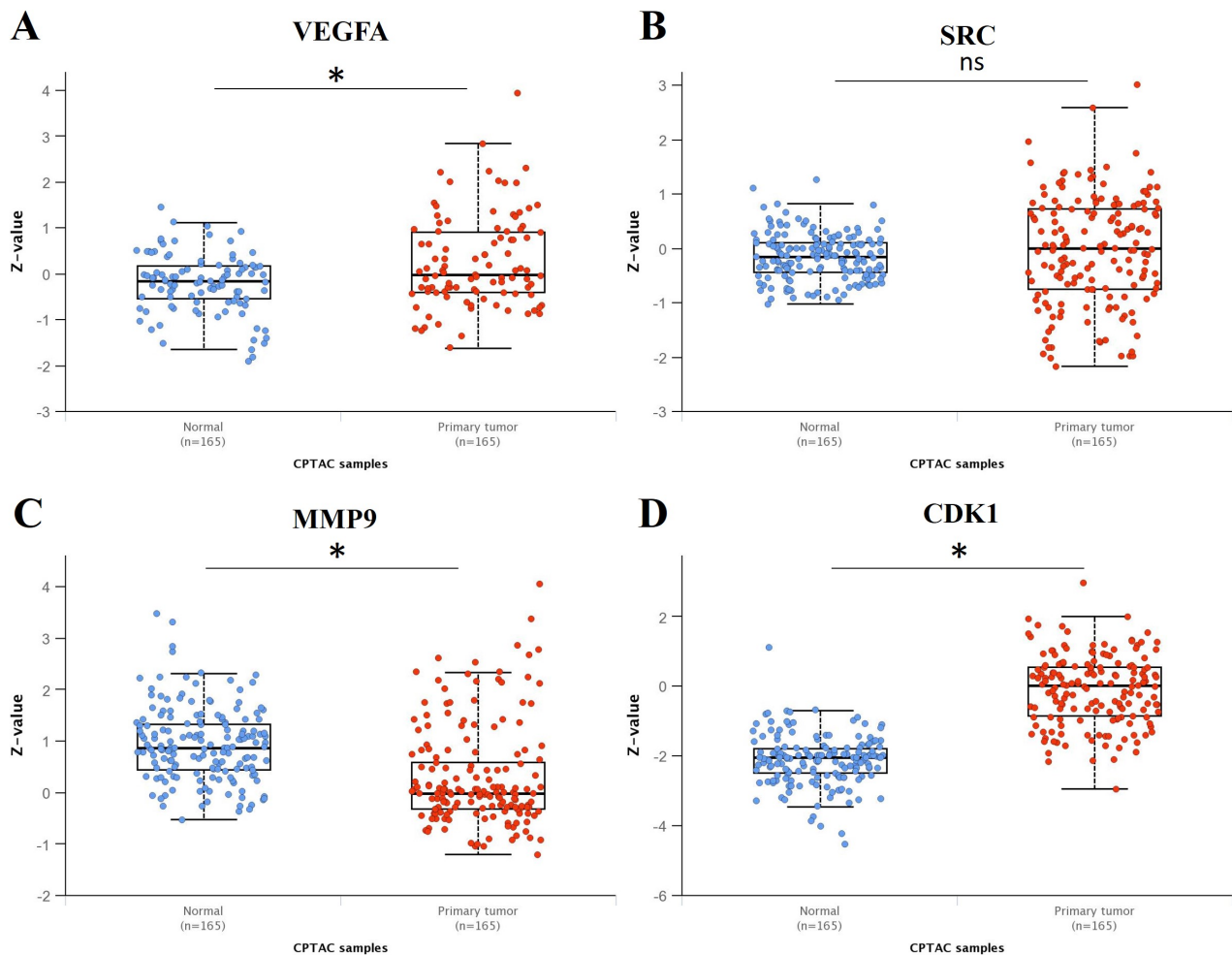


Fig. 9. Analysis of the proteomic expression levels of four genes between the normal samples and primary LIHC tumor samples. (A) VEGFA; (B) SRC; (C) MMP9; (D) CDK1. VEGFA, MMP9, and CDK1 showed significantly different proteomic expression levels between normal ($n = 165$) and primary tumor samples ($n = 165$). $*p < 0.05$ was defined as statistically significant. Not significant (ns) was detected for the expression of SRC protein. Z-values represent the standard deviations from the median across normal or LIHC samples. The log2 spectral count ratio values of CPTAC samples were first normalized within each sample and then normalized across samples.

Firstly, we examined the correlation between the expression levels of the four key hub genes and the tumor-infiltrating levels of several immune cells, including B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells in LIHC. The results indicated that the expression levels of genes *VEGFA*, *SRC*, *MMP9*, and *CDK1* were significantly correlated with the tumor-infiltrating levels of different types of immune cells in LIHC (Fig. 10).

To determine the significance of the immune checkpoint markers (*PDCD1*, *CD274*, *CTLA4*, and *CD47*) in HCC, the mRNA and proteomic expression levels in clinic LIHC primary tumor ($n = 371$) and non-tumor control samples ($n = 50$) were analyzed using the data from the TCGA database. The results demonstrated that checkpoint markers *PDCD1*, *CTLA4*, and *CD47* showed significantly higher mRNA expression levels in LIHC primary tumors com-

pared with the non-tumor samples. In contrast, *CD274* showed a decreased trend ($p = 4.95 \times 10^{-1}$) in mRNA expression levels in LIHC primary tumor samples compared with non-tumor control samples (Fig. 11). The CPTAC database was applied to analyze the proteomic expression levels of immune checkpoints. The results revealed that both proteins encoded by *CD274* and *CD47* showed significantly lower expression levels in LIHC primary tumors ($n = 165$) compared with non-tumor samples ($n = 165$) (Fig. 12). However, the expression levels of proteins encoded by *PDCD1* and *CTLA4* were not attainable using this database.

Then, we evaluated the co-expression levels of four identified genes *VEGFA*, *SRC*, *MMP9*, and *CDK1* with the genes of well-known immune checkpoints that play an important role in immunotherapy, including *CD274*, *PDCD1*,

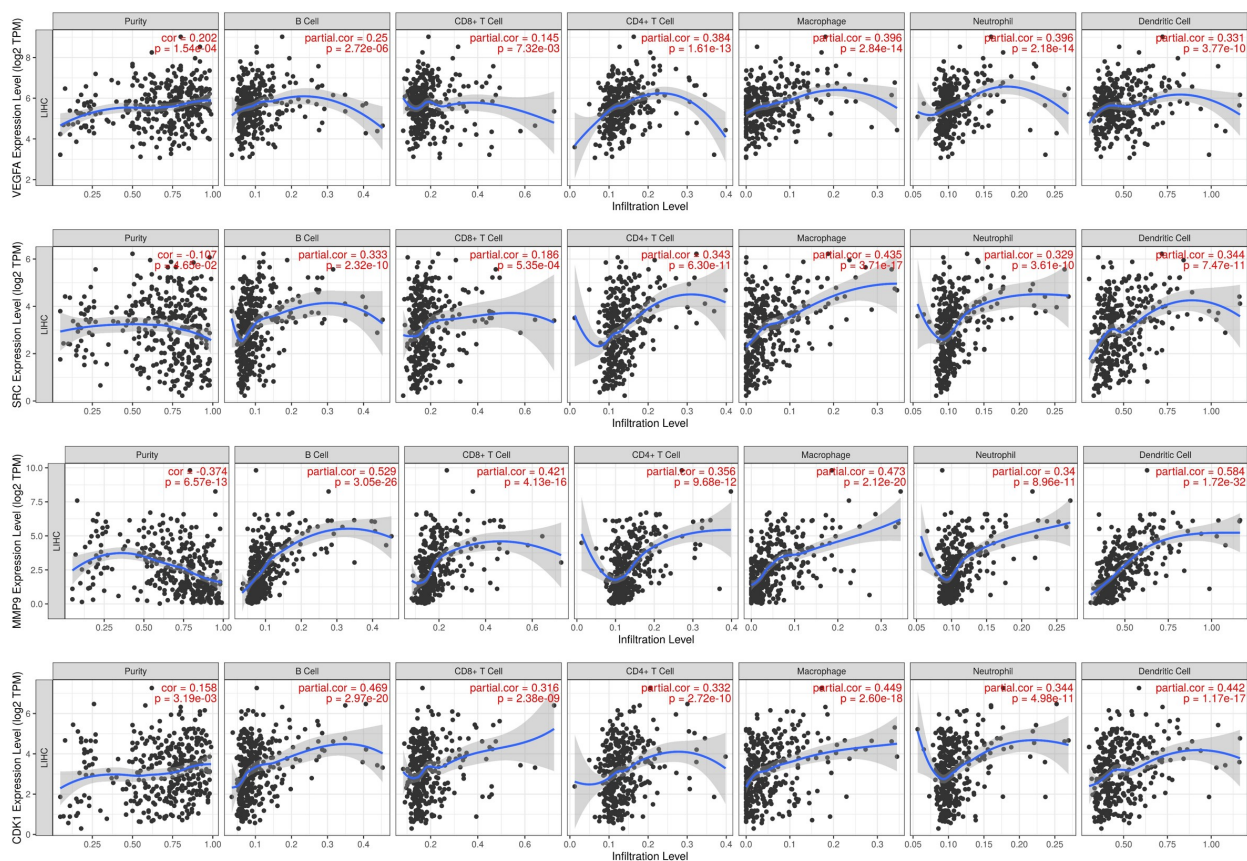


Fig. 10. The correlation of the gene expression levels and the levels of tumor-infiltrating immune cells in LIHC. The expression levels of genes *VEGFA*, *SRC*, *MMP9*, and *CDK1* were significantly correlated with the tumor-infiltrating immune cell levels of B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells in liver cancer. p -value < 0.05 indicates statistical significance.

CTLA4, and *CD47*. The results showed *VEGFA*, *CDK1*, *MMP9*, and *SRC* had statistically significant co-expression levels with genes *CD274*, *PDCD1*, and *CD47*, respectively. *VEGFA*, *CDK1*, and *MMP9* showed significant co-expression levels with *CTLA4*. However, there was no significant co-expression level between *SRC* and *CTLA4* (Fig. 13).

3.9 Molecular Docking for the Interaction of Bilobetin with four Key Hub Genes

To investigate the potential molecular mechanisms of bilobetin-targeted genes *VEGFA*, *CDK1*, *MMP9*, and *SRC*, we applied a computer-aided approach to perform the molecular docking between bilobetin and proteins encoded by four key hub genes, respectively. The results demonstrated there was an optimistic binding affinity between bilobetin and proteins VEGFA (−8.8 kcal/mol), CDK1 (−10.0 kcal/mol), MMP9 (−11.0 kcal/mol), and SRC (−10.6 kcal/mol), respectively. The detailed docking information is listed in a table (Table 1).

The formation of hydrogen bonds between bilobetin and proteins VEGFA, CDK1, MMP9, and SRC were tested and displayed with the labeled distances (Fig. 14). In the

bilobetin-VEGFA binding complex, bilobetin formed hydrogen bonds with residues GLU-30, LEU-32, GLN-37, and ARG-56 of the VEGFA protein. In bilobetin-SRC binding complex, residues GLY-279, GLY-276, and SER-345 of the SRC protein contributed to the formation of hydrogen bonds. Residues ALA-189, ALA-191, GLU-227, GLN-108, and PHE-107 of the MMP9 protein were involved in the formation of hydrogen bonds in the bilobetin-MMP9 binding complex. Residue TYR-15 of the CDK1 protein formed a hydrogen bond with the bilobetin molecule during the interaction (Fig. 14).

In addition to the hydrogen bonds, the hydrophobicity interaction between bilobetin and proteins also contributed to the binding affinity and stability of the complexes (Fig. 15).

In addition, other interactions between bilobetin and target proteins include van der Waals, Pi-sigma, Pi-Pi stack, Pi-Pi t-shaped, Pi-alkyl, Pi-cation, Pi-anion, Pi-sigma, amide-Pi stack, alkyl, and covalent bonds, which contributed to the stability of the complexes (Fig. 16).

To further evaluate the drug-likeness of bilobetin, we performed ADME analysis using SWISSADME bioinformatic tool, a commonly used tool in drug discovery. The

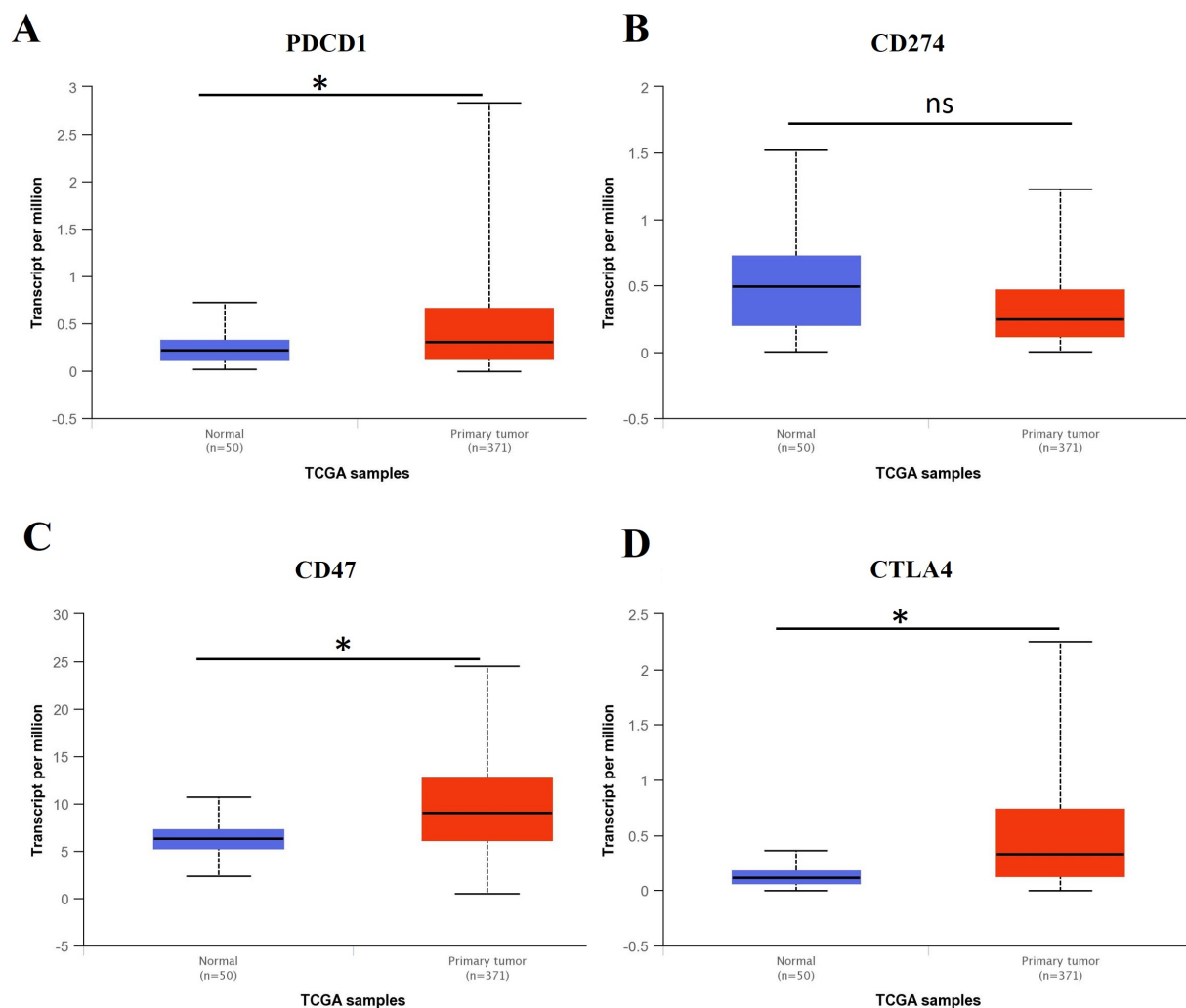


Fig. 11. The expression level of immune checkpoint biomarkers in LIHC tumor and non-tumor control samples. The mRNA expression levels of (A) *PDCD1* ($p = 3.01 \times 10^{-8}$), (B) *CD274* ($p = 4.95 \times 10^{-1}$, ns: not significant), (C) *CD47* ($p = 9.99 \times 10^{-12}$), and (D) *CTLA4* ($p = 2.89 \times 10^{-12}$) in LIHC primary tumor samples compared with non-tumor control samples. * p -value < 0.05 indicates statistical significance.

results suggested that the bilobetin meets the requirements of the drug-likeness according to the filter applied by the Lipinski filter (Pfizer) (Table 2).

4. Discussion

Liver cancer ranks at the top of cancer-associated deaths with no effective therapeutic strategies. Early diagnostic biomarkers and the discovery of effective treatments for LIHC are urgently needed. Bioinformatic analysis empowers the understanding of the pathogenesis of cancers and the discovery of disease-related biomarkers [46,47]. Computer-aided drug discovery is a favorable strategy for drug development and mechanistic investigation [48]. In this study, we comprehensively integrated these two powerful research approaches to investigate and explore potential therapeutic agents for liver cancer. Our results have demonstrated that bilobetin is a potent thera-

peutic target for HCC/LIHC via interaction with four key hub targets, including VEGFA, SRC, MMP9, and CDK1, which are associated with immune cell tumor infiltration and co-expression of immune checkpoints.

Among the molecular targets of bilobetin, 16 genes were identified to play essential roles in the signaling pathway of reactive oxygen species (ROS) ($p = 2.09 \times 10^{-8}$, Fig. 3), including *CBR1*, *PTPN1*, *SRC*, *AKR1C1*, *AKR1C3*, *AKR1A1*, *AKR1C2*, *AHR*, *PIK3R1*, *AKR1C4*, *PTK2*, *VEGFA*, *CYP1B1*, *NOX4*, *AKT1*, and *MET*. Among the genes, 7 out of 16 genes (*PTPN1*, *SRC*, *AKT1*, *PIK3R1*, *MET*, *PTK2*, *VEGFA*) were further identified as hub genes that contribute to the ROS signaling pathway ($p = 7.60 \times 10^{-6}$). Among the bilobetin-targeted genes, five genes *BCL2A1*, *PARP1*, *AKT1*, *PIK3R1*, and *MCL1* were involved in the apoptosis signaling pathway ($p = 0.066148$), and four including *PARP1*, *AKT1*, *PIK3R1*, and *MCL1* were further

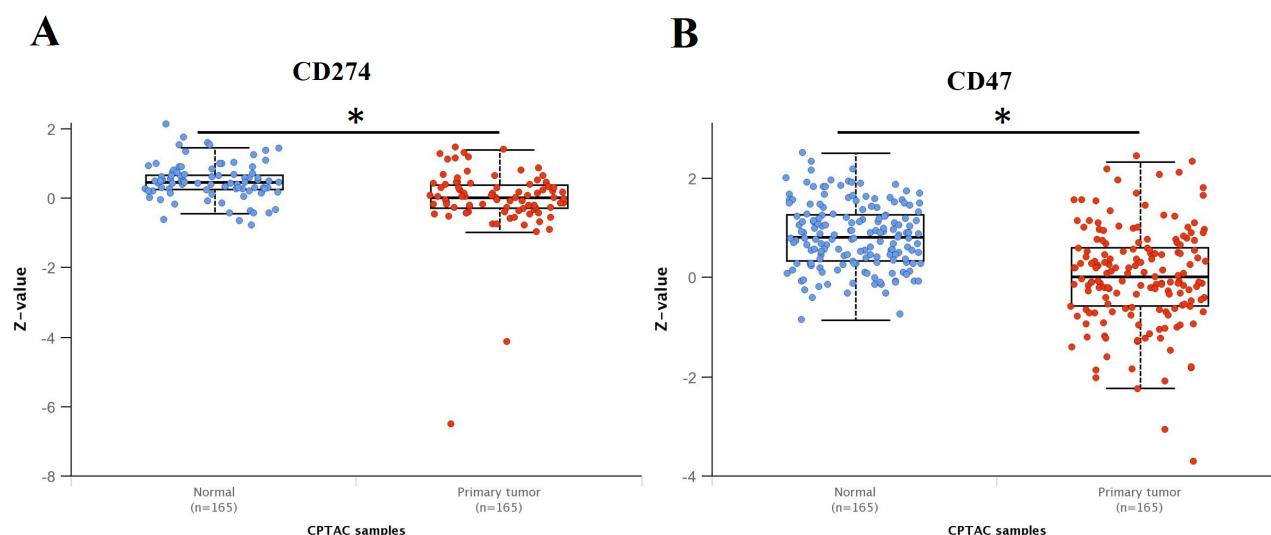


Fig. 12. The expression of proteins encoded by genes *CD274* and *CD47* in the CPTAC database. The protein expression level results revealed that both (A) *CD274* and (B) *CD47* proteins showed significantly lower expression levels in LIHC primary tumors ($n = 165$) compared with non-tumor control samples ($n = 165$). * p -value < 0.05 indicates statistical significance.

Table 2. Evaluation of the pharmacokinetics, drug-likeness, and medicinal chemistry of bilobetin.

Parameters	Physicochemical properties
Number of rotatable bonds	4
Number of H-bond acceptors	10
Number of H-bond donors	5
Molar Refractivity	151.44
TPSA	170.8
Log Po/w calculation methods and lipophilicity	
iLOGP	3.39
XLOGP3	5.36
WLOGP	5.44
MLOGP	0.44
Silicos-IT	5.16
Average value	3.96
Pharmacokinetics	
GI absorption according to the white of boiled egg	Low
BBB permeant according to the yolk of boiled egg	No
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	Yes
CYP2D6 inhibitor	No
CYP3A4 inhibitor	No
log Kp (skin permeation, cm/s)	-5.86
Medicinal Chemistry	
PAINS #alerts	0
Synthetic Accessibility	4.35
Druglikeness	
Lipinski Filter	Yes
Bioavailability Score	0.55

Note: TPSA, topological polar surface area; CYP, cytochrome P450; GI, gastrointestinal; P-gp, P-glycoprotein substrate; PAINS, pan-assay interference structures.

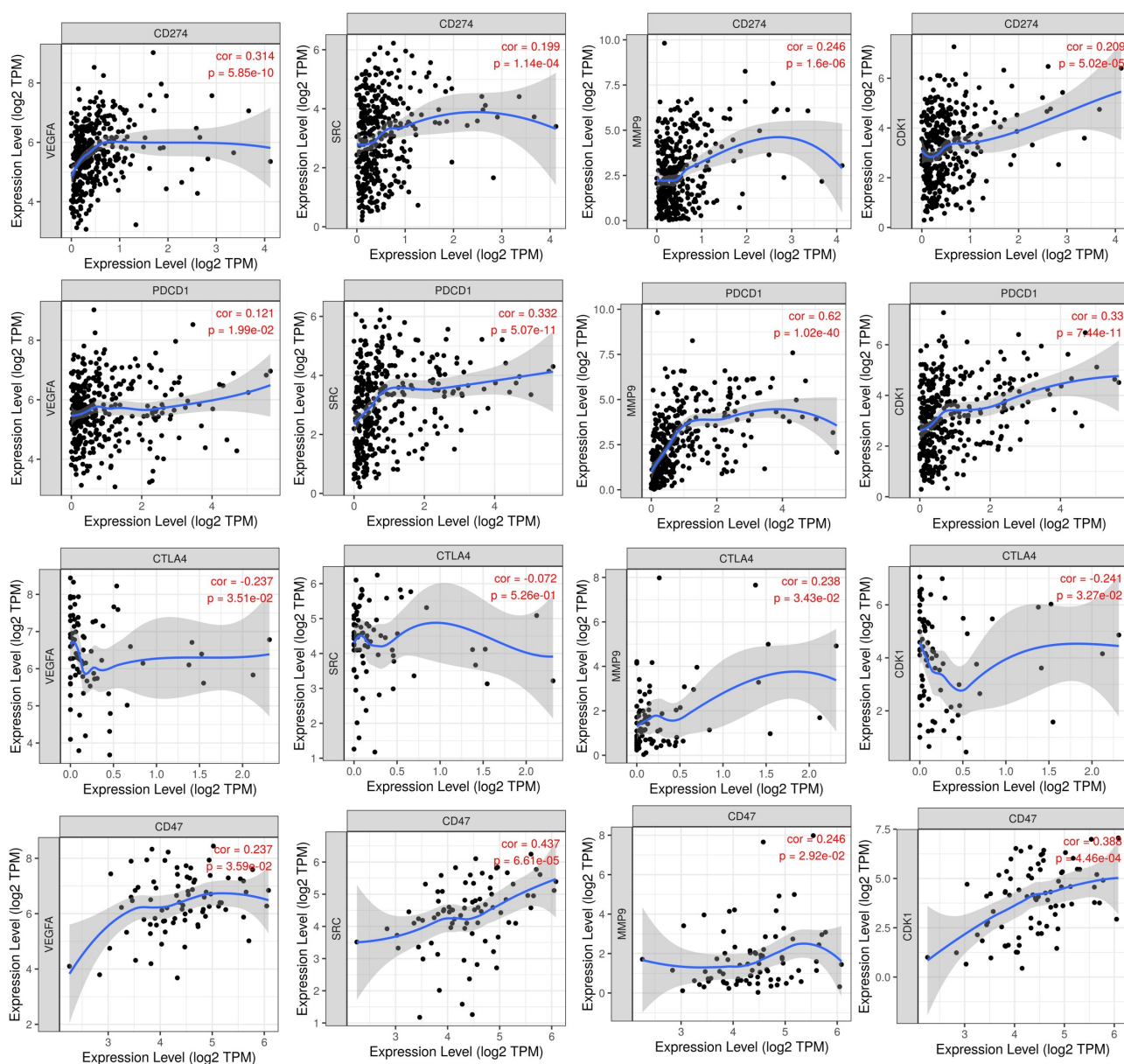


Fig. 13. The co-expression levels of four key hub genes *VEGFA*, *SRC*, *MMP9*, and *CDK1* with the immune checkpoint genes *CD274*, *PDCD1*, *CTLA4*, and *CD47*. With the exception of the gene *SRC* which did not indicate significant correlation with *CTLA4*, all other genes *VEGFA*, *MMP9*, and *CDK1* showed statistically significant co-expression levels with genes *CD274*, *PDCD1*, *CTLA4*, and *CD47*, respectively. *SRC* showed a significant co-expression level with genes *CD274*, *PDCD1*, and *CD47*. p -value < 0.05 indicates statistical significance.

identified as hub genes that participate in the signaling pathway of cell apoptosis ($p = 0.003554$). These results suggest that the regulation of cellular production of ROS and cell apoptosis might also be the acting mechanism of bilobetin.

VEGFA protein plays a critical role in the growth of tumor-associated blood vessels and vascular permeability, accelerating tumor cell invasion [49–51]. Studies also found that the circulating levels of VEGFA can serve as a prognostic biomarker for the prediction of survival outcomes of LIHC patients [52,53]. In addition, VEGFA derived from hepatocytes can promote the development

of liver diseases, such as non-alcoholic fatty liver disease which becomes a leading cause of HCC [54]. Currently, the investigation and development of VEGFA inhibitors as therapeutic options against cancers including LIHC are gaining much more attention [55,56]. In this study, we found *VEGFA* is one of the bilobetin-targeted key hub genes. Surprisingly, our results have revealed that the mRNA expression levels of *VEGFA* were significantly lower in LIHC tumors compared with the normal samples. However, when we examined the proteomic data, we found the proteomic level of VEGFA was significantly increased

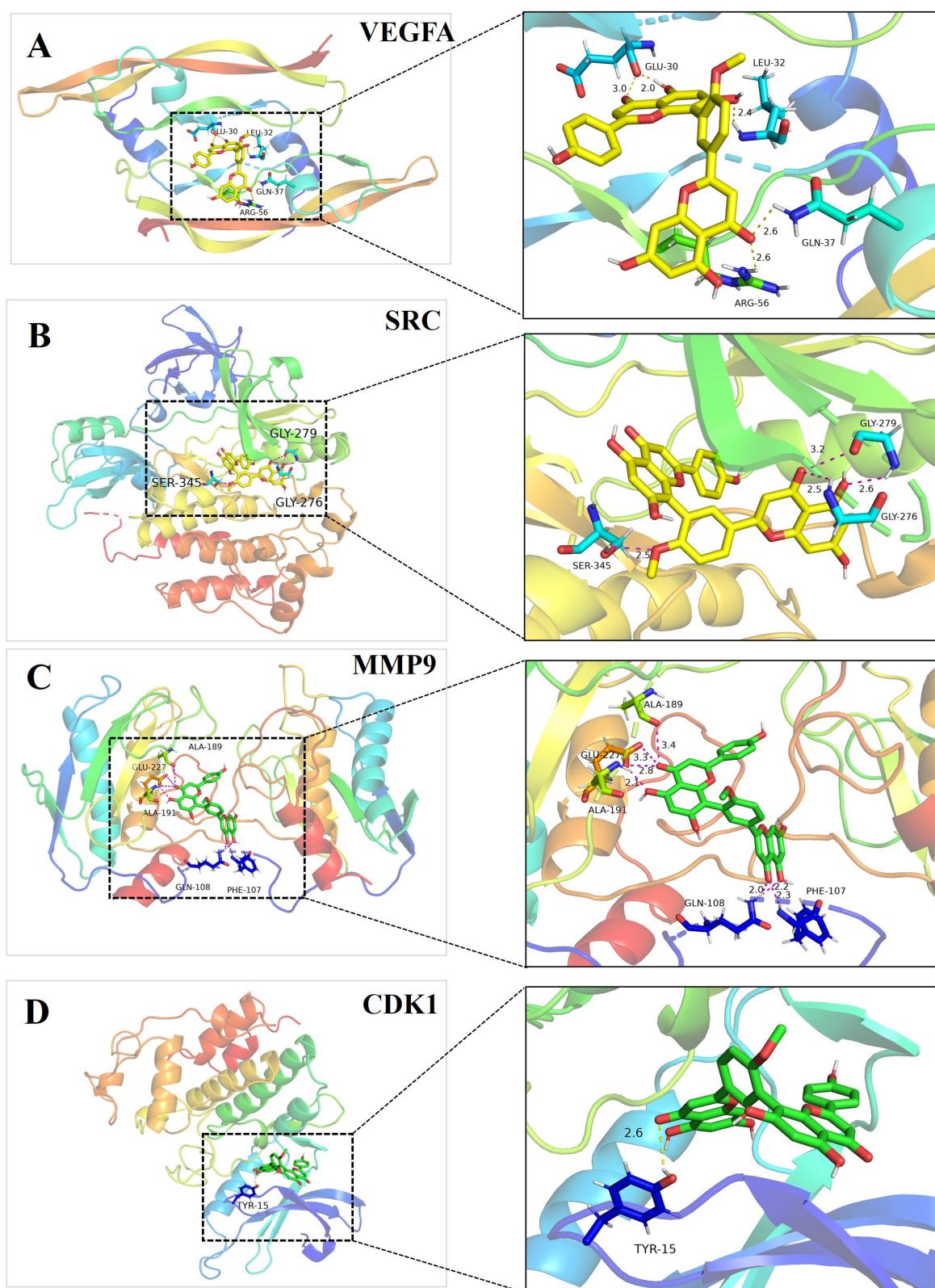


Fig. 14. The formation of hydrogen bonds in the binding complex of bilobetin and proteins encoded by target genes. The key residues that contributed to the formation of hydrogen bonds and the distance between bilobetin and residues were measured and labeled. Left: the overall structure of proteins used for molecular docking. Right: the detailed interaction between bilobetin and its target proteins. (A) VEGFA; (B) SRC; (C) MMP9; (D) CDK1.

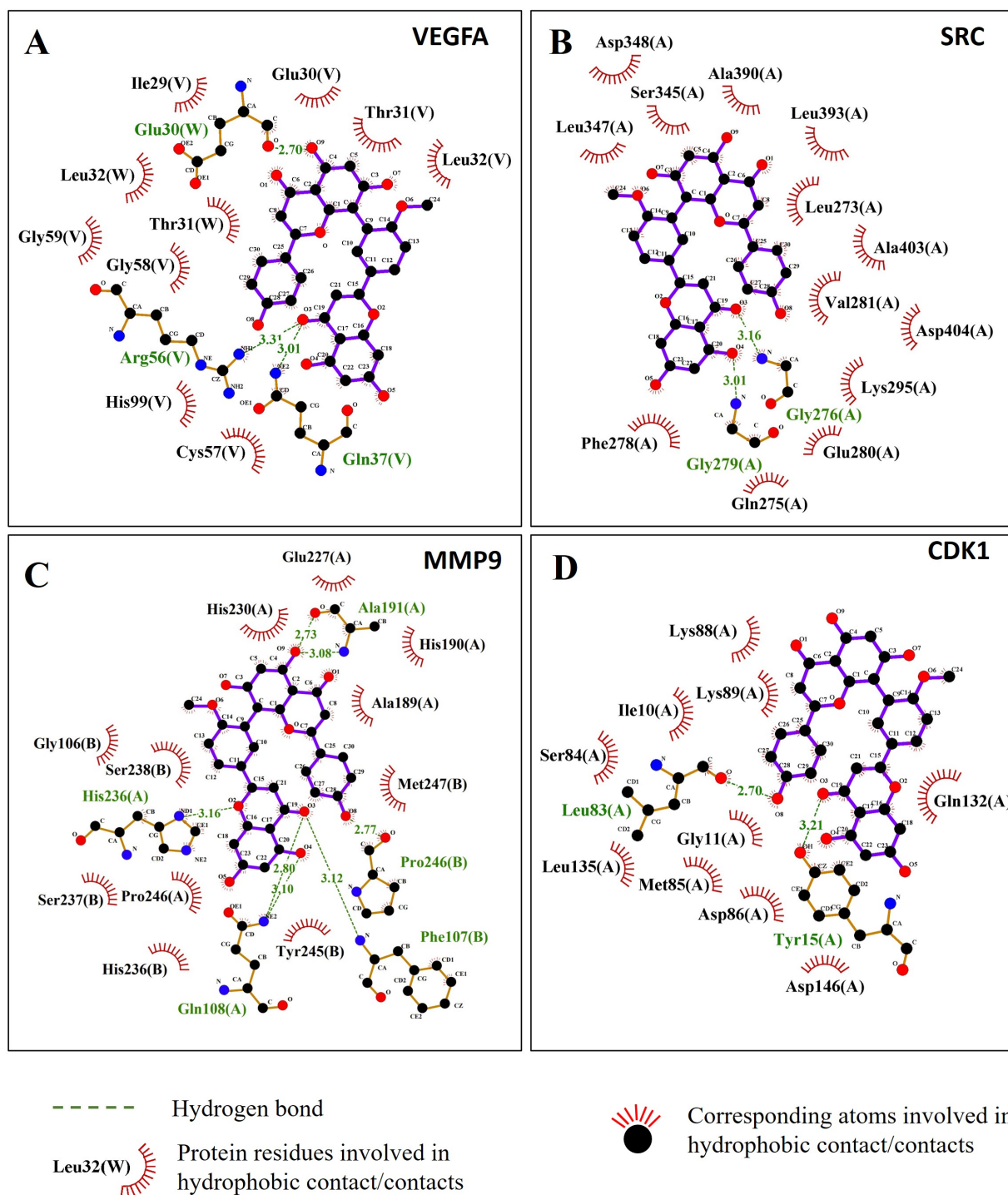


Fig. 15. The 2D interaction results showed hydrophobic interactions between bilobetin and four target proteins. (A) VEGFA; (B) SRC; (C) MMP9; (D) CDK1.

in LIHC tumors compared with normal samples. Consistent with other studies [53,57,58], we also found there is a significant association between the expression levels of *VEGFA* and the clinical survival outcomes of LIHC patients. Therefore, targeting *VEGFA* using bilobetin as a potential therapeutic strategy for HCC treatment can be further investigated.

SRC plays a significant role in cancer development [59–62]. In our study, we have demonstrated that *SRC* is one of the identified bilobetin-targeted hub genes. The mRNA expression levels of *SRC* were significantly different from LIHC tumor samples compared with normal samples, which were significantly associated with survival outcomes of LIHC patients. The expression levels of *SRC* also showed a significant association with tumor-related im-

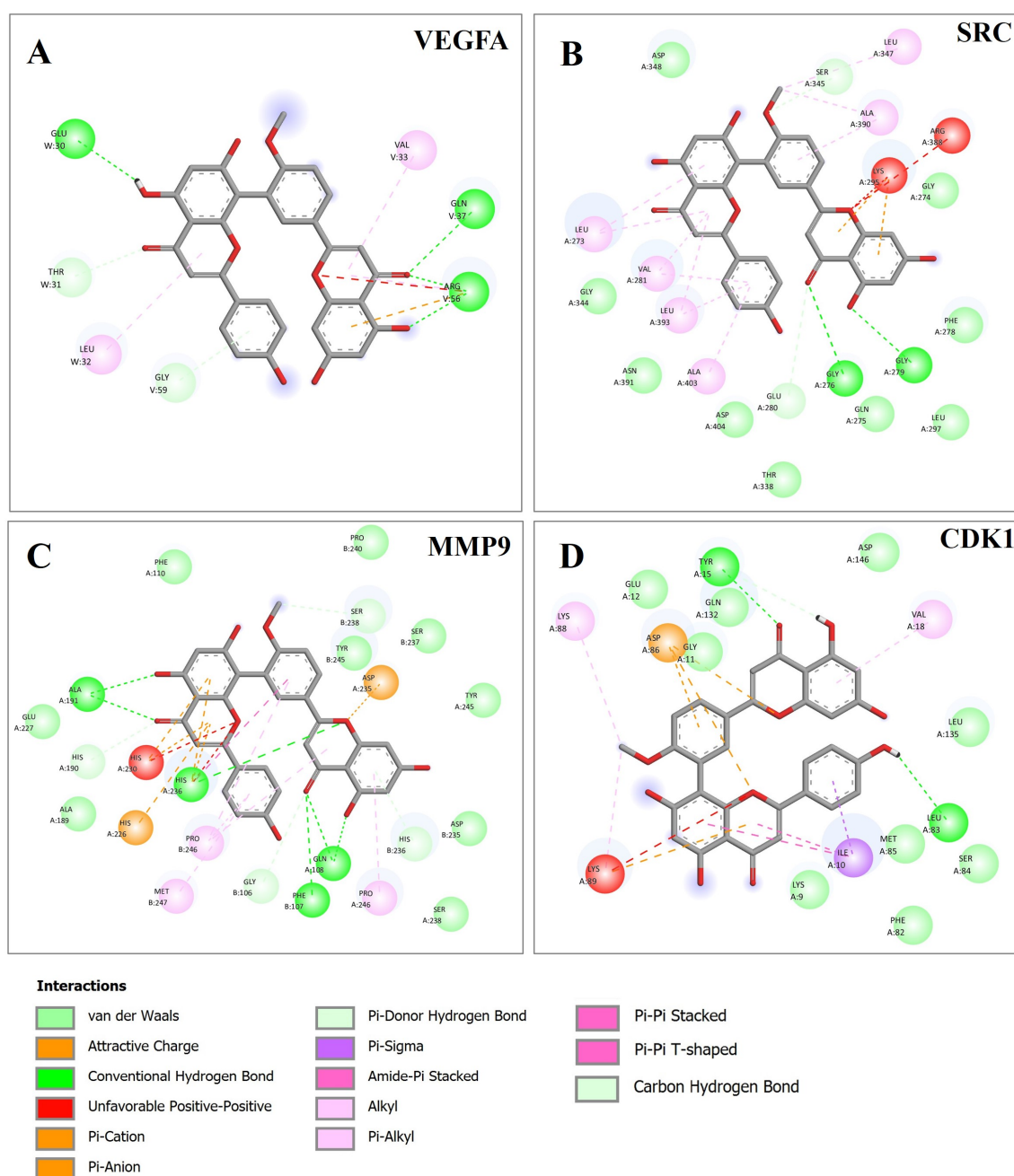


Fig. 16. The 2D interaction analysis results of bilobetin and the target proteins. The figure presents all the interactions between bilobetin and its target proteins that contribute to the formation of the protein-ligand complexes. (A) VEGFA; (B) SRC; (C) MMP9; (D) CDK1.

immune cell infiltration and the expression of immune checkpoints. However, the proteomic expression levels of SRC do not show statistical significance between normal and tumor samples, which requires further investigation. The phosphorylation of SRC proteins also plays a pivotal role in liver disease [63]. Therefore, the mRNA expression levels and phosphorylated status of SRC proteins are required to evaluate their role in LIHC.

Consistent with our previous analysis results (unpublished), *MMP9* was identified as an important gene in LIHC, as there is a significant association between its

mRNA and protein expression levels with the survival outcomes of LIHC patients, as well as tumor-infiltrating levels of immune cells. In this study, we have demonstrated that the bioactive compound bilobetin has the best binding affinity (−11.0 kcal/mol) with *MMP9* compared with VEGFA (−8.8 kcal/mol), SRC (−10.6 kcal/mol), and CDK1 (−10.0 kcal/mol). Therefore, our study provides an optimistic insight for further evaluating bilobetin as a potential therapeutic agent for LIHC treatment by regulating the expression of *MMP9*, which plays a key role in cancer development [64,65].

CDK1 is another key hub gene identified from 20 bilobetin-targeted hub genes. In line with other research findings [66–68], our results also revealed that both the mRNA and proteomic levels of gene *CDK1* showed significantly higher expression levels in LIHC tumors compared with normal samples. In addition, the expression of *CDK1* was significantly associated with the survival outcome of LIHC patients and tumor-associated immune cell infiltration, as well as the expression levels of immune checkpoint-related biomarkers. The molecular docking results also showed that bilobetin can be used as a compound template for designing new drugs for LIHC treatment.

Drug development is a long process, and comprehensive or integrated research approaches such as bioinformatic analysis and computer-aided methods could bring some advantages to promote the process. Overall, our study shows that bilobetin can target multiple targets that influence liver cancer development and progression, which holds great value in the improvement of treatment efficacy and reduction in the side effect.

5. Conclusions

In this study, we explored the potential of bilobetin as a LIHC therapeutic agent based on comprehensive bioinformatic analysis and computer-aided drug discovery approaches. Although one of the most important drug discovery processes, further steps such as modification, optimization, examination of toxicity or side effects, and *in vitro* and *in vivo* experiments are necessary and should be carefully evaluated. The functionality, delivery method, and treatment efficacy should also be further studied.

Availability of Data and Materials

The data involved in this study were obtained from publicly available online database. The online sites include the following: Swiss Target Prediction: <http://swisstargetprediction.ch/>; GEPIA: <http://gepia.cancer-pku.cn/>; TCGA: <https://portal.gdc.cancer.gov/>; TIMER: <http://timer.cistrome.org/>; UALCAN: <https://ualcan.path.uab.edu/>; Human Protein Atlas: <https://www.proteinatlas.org/>; PDB: <https://www.rcsb.org/>.

Author Contributions

CZ and MY designed the research study. CZ, YS, SL, and MY performed the research. CZ, YS, SL, and MY analyzed the data. CZ, YS, SL, and MY wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

Given his role as Guest Editor, Ming Yang had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Jian Zhang and Ricardo Jorge Pinto Araujo. The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2812360>.

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