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Original Research Article

Bioinformatics and experimental validation of mechanisms underlying lenvatinib therapy for hepatocellular carcinoma

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Abstract

Purpose: *To investigate and validate the therapeutic targets of lenvatinib in hepatocellular carcinoma (HCC), using bioinformatics and in vitro experiments.*
Methods: *The potential core targets of lenvatinib were identified through screening and analysis of various databases. The core targets were validated using in vitro experiments.*
Results: *The results showed that lenvatinib significantly affected pro-survival signals of MAPK signaling pathway which activate and regulate a range of cellular activities. Molecular docking revealed that key proteins may be the key targets of lenvatinib in its systematic anti-tumor effect on HCC. Results from quantitative reverse transcription-polymerase chain reaction (qRT-PCR) revealed that lenvatinib regulated the expressions of key genes associated with HCC.*
Conclusion: *The findings of this investigation provide a new insight for further development of lenvatinib as a drug for HCC treatment.*

Keywords: *Lenvatinib, Hepatocellular carcinoma, Bioinformatics, Molecular docking, Network pharmacology, Protein-protein interaction*

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INTRODUCTION

Statistics show that in 2018, liver cancer was ranked the sixth largest cancer worldwide, as it accounted for 8.2 percent of the total cancer fatality [1,2]. Liver cancer is classified into primary and secondary types. The more common type of primary liver cancer, i.e., hepatocellular carcinoma (HCC), constitutes 90 percent of primary liver cancers and is associated with high mortality [3]. So far, the most popular treatment strategy for HCC has been comprehensive therapy based on surgical resection and adjuvant therapies such as radiotherapy and chemotherapy.

However, the mechanism underlying the occurrence and progression of HCC is related to a complex network of interactions amongst multiple genes, multiple reactions, and multiple signaling pathways. Thus, the recurrence rate of hepatocellular carcinoma after radical resection ranges from 40 to 50 % within 3 years, and the recurrence rate of metastasis is 60 – 70 % in 5 years [4]. Perhaps due to being difficulty in early diagnosis, the majority of HCC patients are

diagnosed at the terminal stage with evidence of intra- and extrahepatic metastases or portal invasion, at which point, the opportunity for surgery is missed, thereby making the malignancy a serious threat to life [5-7].

Lenvatinib, an oral tyrosine kinase inhibitor, obstructs poly-molecular channels, and its major targets are VEGFR1-3, RET, FGFR1-4, c-KIT, PDGFR-α, and PDGFR-β. Besides, it exerts anti-tumor cell proliferation and anti-angiogenic effects [8,9]. Clinical studies have revealed that the survival rates of whole untreated terminal HCC cells exposed to Lenvatinib were comparable to that resulting from treatment with sorafenib [10]. Although the intensive study of kinases as drug targets has brought great benefits to HCC patients, their development in oncology remains limited and challenging due to the complexity of molecular signaling in tumors [11].

In the present study, a network pharmacology approach was used to find out whether there are still new targets for Lenvatinib in HCC, as well as mechanisms of action of its anti-tumor effects. The network pharmacology involved predicting and searching for Lenvatinib-related targets, identifying core proteins and functions using the network methods of protein-protein interaction (PPI), and confirming key proteins through bound docking. In addition, the results of bound docking were used for trials on cells *in vitro* to determine the potential effects of Lenvatinib on HCC. Figure 1 demonstrates the route map of the research in detail.

This study is the first investigation involving the application of network pharmacology in the determination of the efficacy and mechanism of action of Lenvatinib. The results may be highly beneficial for the development and application of Lenvatinib for clinical treatment of HCC.

EXPERIMENTAL

Materials

The Chemical Substance Registration Number (CAS) of Lenvatinib is 942407-57-0. Lenvatinib has a molecular formula of $C_{21}H_{19}ClN_4O_4$ and a molecular weight of 426.9 g/mol. It is incorporated into GeneCards database of website https://www.genecards.org/. Lenvatinib is also included in the website https://pubchem.ncbi.nlm.nih.gov/ belonging to PubChem database. It is also contained in the literature of PubMed Central in NCBI database whose website is http://www.ncbi.nlm.nih.gov/pubmed/, and it appears in pharmacophore matching and potential identification of targets of PharmMapper database with site URL of http://www.lilab-ecust.cn/pharmmapper/. The site URL of Drugbank (https://go.drugbank.com/) is one collection that covers the term Lenvatinib.

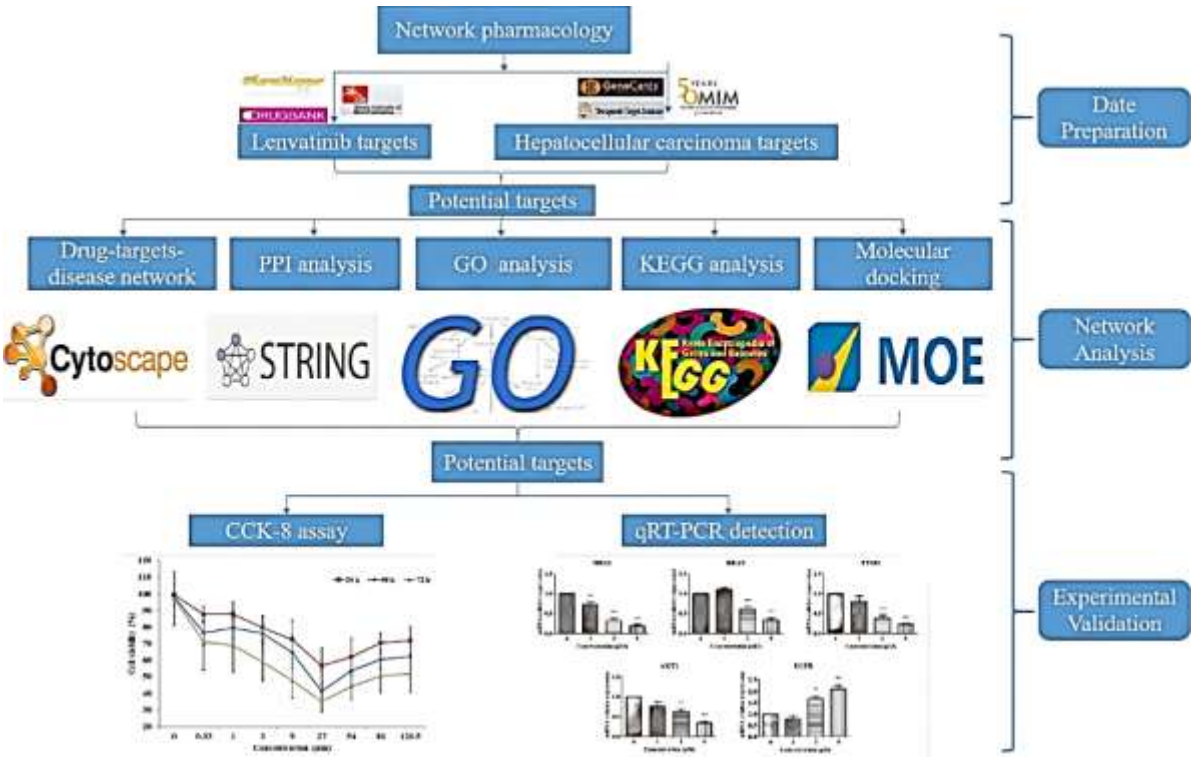

Figure 1: The roadmap of this study

It is also recorded in Swiss TargetPrediction database (http://www.swisstargetprediction.ch/), GeneCards database (https://www.genecards.org/, TTD database (http://db.idrblab.net/ttd/), OMIM database (http://omim.org/), Cytoscape 3.6.0 software for network topological property analysis (http://www.cytoscape.org/), STRING database (http://string-db.org/), the Biological Information Annotation database DAVID (version: 6.8) (https://david.ncifcrf.gov/); Kyoto Encyclopedia of Genes and Genome (KEGG) database (http://www.genome.jp/kegg/), and Protein Data Bank database (https://www.rcsb.org).

The software was run under the Windows operating system platform with the processor Inter(R) Core (TM) i3-330M CPU@2.13GHz, 64-bit operating system.

Lenvatinib (E7080) was purchased from Aladdin Biochemical Technology Company (Shanghai, China); both DEME and FBS were bought from Gibco (Life Technologies Corporation, USA); CCK-8 kit was purchased from Beyotime Biotechnology Group (Shanghai, China); Hiscript II cDNA reverse transcription instruments were obtained from Vazyme Biotech Co. Ltd (Nanjing, China); Gene-specific primers and SYBR® Premix Ex Taq™ Ⅱ (Tli RNaseH Plus) were purchased from TaKaRa Bio, Inc., (Japan).

Ligand preparation

Lenvatinib was used as a ligand, and structural data were downloaded from the PubMed organic small molecule bioactivity database to obtain its planar and three-dimensional (3D) chemical structure diagrams.

Prediction of potential targets for lenvatinib

The potential targets for lenvatinib were obtained in two ways. In the first approach, known targets were obtained by querying the NCBI databases PubMed Central for literature and Drugbank. In the second approach, key targets were predicted by querying the PharmMapper and the Swiss TargetPrediction.

Collection and organization of known hepatocellular carcinoma-related genes

The potential target genes of HCC were determined using GeneCards, TTD, and OMIM databases. The relevant targets of HCC were searched with the keywords 'hepatocellular carcinoma, and the potential target genes were counted.

Network establishment

The potential target proteins of lenvatinib and the annotated gene names were entered into online analysis database STRING of the protein-protein interaction (PPI). The protein interactions of each drug target network were analyzed (species: *Homo sapiens* and confidence score > 0.97). The results from the analyses of PPI network were combined with the Cytoscape software (version 3.6.0) to graphically showcase, analyze and edit. In addition, Cytoscape plug-in 'CytoHubber' was used to obtain an intensity score for the interactions of the proteins based on a degree score.

Related pathways and annotation analysis

The correlation between target proteins and the functions of enrichment genes was investigated to study the possible biological pathways used by lenvatinib, as well as the correlation between targets. First of all, the data from David 6.8 database were input in gene symbol format, followed by an analysis of Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway annotation. Based on *p*-value less than 0.05 or *p*-value less than 0.01, human species were selected for analysis of important channels.

Using statistical analysis, GO function enrichment and KEGG approaches with *p*-value less than 0.01 were worked out. The GO annotation analysis involved cellular components, biological processes, and molecular functions.

Analysis of biological processes

To further understand the biological relevance of candidate targets of lenvatinib against HCC, biological processes were analyzed using a Cytoscape plug-in software, 'ClueGo'. It was effective for screening factors under the condition of *p*-value less than 0.05.

Prediction of forward molecular docking between lenvatinib and important target proteins

The above analysis was used to screen for signaling pathways related to hepatocellular carcinoma, after which the target protein nodes in the target pathways were identified, and the three-dimensional (3D) structure charts of the targets were downloaded from the Protein Data Bank database accordingly. The 3D structure charts of chemical compounds were obtained through the PubChem database in SDF format.

The docking software, Molecular Operating Environment (MOE, 2019.0102), was deployed for conducting positive bound docking of lenvatinib, as well as its targets.

Pharmacological verification

Cell lines

Homo sapiens hepatocyte lineage HuH-7 cells were purchased from Fenghui Biotechnology Group (Hunan, China). The HuH-7 cells were cultured at a temperature of 37 ^{o}C in high-glucose DMEM supplemented with 10 percent FBS, penicillin (100 units/mL), and streptomycin (100 µg/mL) in a 5 % carbon dioxide incubator. Using an inverted microscope, the cells were monitored and passaged when the cell density in the flask reached 85 percent. Before 85 % confluence, the cell culture medium in the 75-cm^2 flask was replaced every 24 h.

Measurement of cell survival rate

The HuH-7 cells were seeded in a 96-well plate at a density of 5000 cells per well, with 100 µL of vehicle/well. Wells without Lenvatinib served as control. After 24 h, different concentrations of Lenvatinib (0.5, 1, 3, 9, 27, 54, 81, and 121.5 µM) were used to pretreat the cells for 24, 48, and 72 h. The previous medium was replaced in each well with 90 fresh nutrient medium 10 µL of CCK-8 solution. After 2 h of incubation at 37 ^{o}C in a 5 percent carbon dioxide incubator, an enzyme-labeled instrument (Cytation1, Biotek, USA) was used to measure the absorbance value of each well at 450 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After incubating the HuH-7 cells with different concentrations of Lenvatinib (3, 9, and 27 µM) for 48 h, total RNA was extracted from each group of cells using RNA-Quick purification Kit (ES-RN001, YiShan Biotechnology Corporation, Shanghai, China) in accordance with the manufacturer's protocol. The total RNA was reverse-transcribed to complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit. The purity of the extracted nucleic acids was assessed spectrophotometrically using a NanoDrop spectrophotometer (NanoDropTM 2000/2000c, Thermo Fisher Scientific, USA).

Gene-specific primers and SYBR® Premix Ex Taq™ Ⅱ were used for the measurement of the relative mRNA expression levels of selected genes (Akt1, HRAS, TYSM, BRAF, and EGFR) for 40 cycles in an RT-PCR system (CFX96 Touch, Bio-Rad Laboratories, Inc., USA). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with the level of 18S as the endogenous control. The gene-specific primer sequences used are shown in Table 1.

Table 1: Forward and reverse primer sequences for each target gene

Gene	Forward primer	Reverse primer
HRAS	GACGGAATATAA GCTGGTGG	AGGCACGTCTCCC CATCAAT
BRAF	ATGGATCCAGAC AACTGTTCAAAC	AGGTGATTTTGGT CTAGCTACAGT
TYMS	CCTGAATCACATC GAGCCACTG	GCACCCTAAACAG CCATTTCCA
AK	CACAAACGAGGG	GCCATCATTCTTGA
T1	GAGTACATC	GGAGGAAGT
EGFR	TGCACCTACGGA TGCACTG	CGATGGACGGGAT CTTAGGC
18S	GCAATTATTCCCC ATGAACG	GGGACTTAATCAA CGCAAGC

Statistical analysis

The results are shown as mean ± standard deviation (SD) of more than three independent trials. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons tests were used for statistical analysis, while GraphPad Prism 8.0 software package was used for generation of graphs. Statistical significance was assumed at $p < 0.05$.

RESULTS

PPI network framework

A total of 434 targets associated with lenvatinib were screened and predicted from 4 databases, i.e., Drugbank, PubChem, PharmMapper, and SwissTargetPrediction, after merging and removing duplicates. A total of 1556 targets related to HCC were obtained by screening 3 databases, namely GeneCards, TTD, and OMIM, after merging and removing duplicates. As shown in Figure 2 A, based on Venn diagram, a total of 154 key proteins were combined internally using STRING. After removing duplicates, the PPI network contained 154 node proteins and 58 edges. A lenvatinib-target gene-HCC interaction network was constructed (Figure 2 B), and it revealed that Lenvatinib may exert anti-tumor effect on HCC by acting on these target genes. Moreover, ten hub genes, namely GRB2, HRAS, AKT1, SRC, PTPN1, HSP90AA1, MAPK3, MAPK1, MAP2K1, and ATM were analyzed using CytoHubber (Figure 2 C).

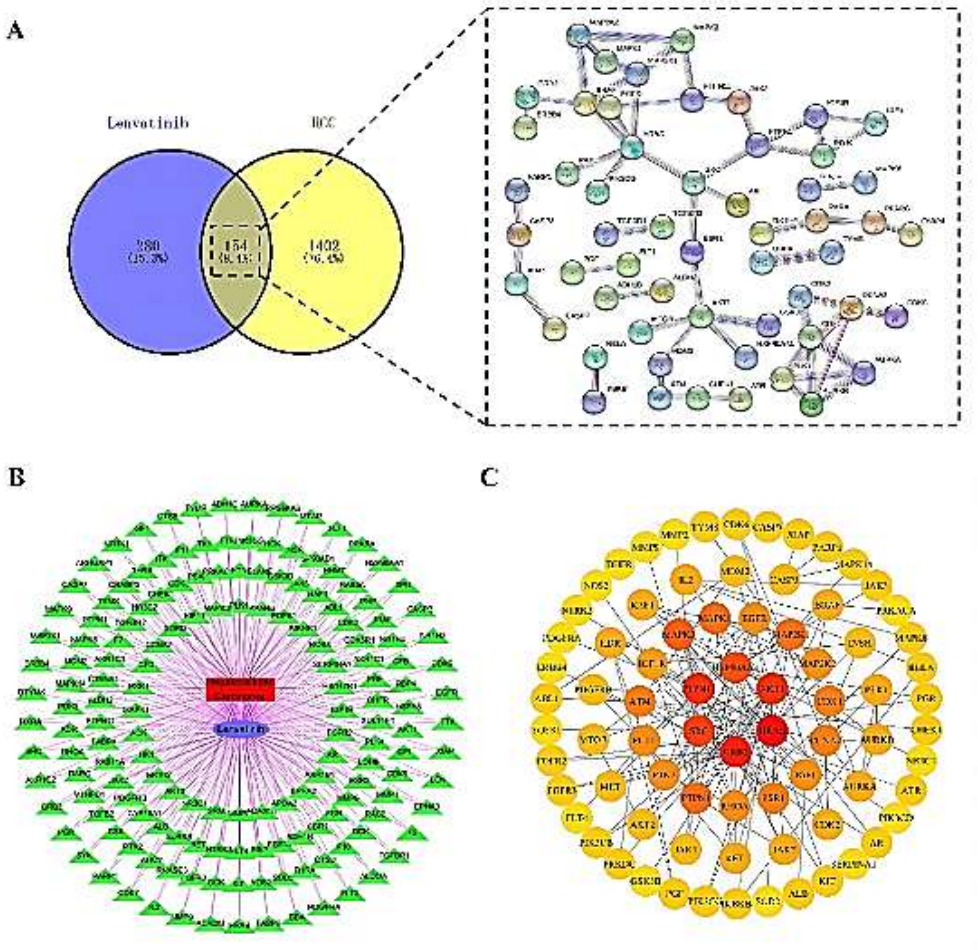

Figure 2: PPI network analysis of lenvatinib in HCC targets. (A) Venn diagram and PPI of Lenvatinib and HCC gene targets. (B) Hub gene network (the colors range from red to yellow, representing the strengths of the genes, from strong to weak, respectively). (C) Network of shared target genes of lenvatinib on HCC (green nodes represented the shared target genes)

Functional enrichment and passage analysis

On importing the disease and drug common key targets from Figure 2 into the David 6.8 database, GO analysis yielded 457 BPs, including peptidyl-tyrosine phosphorylation, protein kinase B signaling, and regulation of protein kinase B signaling; 6 CCs, including membrane raft, membrane microdomain, and secretory granule lumen; 34 MFs, including protein tyrosine kinase activity, protein serine/threonine kinase activity, transmembrane receptor protein tyrosine kinase activity and steroid hormone receptor activity (Figure 3 A). As shown in Figure 3 B, a total of 146 KEGG pathways were involved in enrichment of these pathways. The top 10 signaling pathways were prostate cancer, central carbon metabolism in cancer and mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K)-Akt and Ras signaling pathway, and multiple pathways related to inflammation or cancers.

ClueGo bioprocesses enrichment analysis

As shown in Figure 4, based on ClueGo analysis of effect of lenvatinib when used against HCC, 12 biological processes showed significance of $p < 0.01$. The major areas were positive regulation of MAPK cascade and kinase activity, cardiac muscle tissue growth, mammary gland epithelium development, regulation of receptor signaling pathway via JAK-STAT, and nuclear receptor activity. Biological processes enrichment analysis highlighted many of the key processes and critical factors affected by lenvatinib when used against HCC. The pie chart showed the proportions of the nine major biological processes.

Lenvatinib-hepatocellular carcinoma key network target molecular docking

In this study, molecular docking was used to investigate the binding potential of lenvatinib to key network targets of HCC. As shown in Table

2, the results revealed that the docking scores of lenvatinib with HRas proto-oncogene, GTPase (HRAS), B-Raf proto-oncogene, serine/threonine kinase (BRAF), thymidylate synthetase (TYMS), protein kinase B 1 (AKT1) and epidermal growth factor receptor (EGFR) were less than -15.0, and the binding affinities were the strongest. Figure 5 shows the 3D structure diagram of the complexes formed after docking lenvatinib with the HRAS, BRAF, TYMS, AKT1, and EGFR ligands.

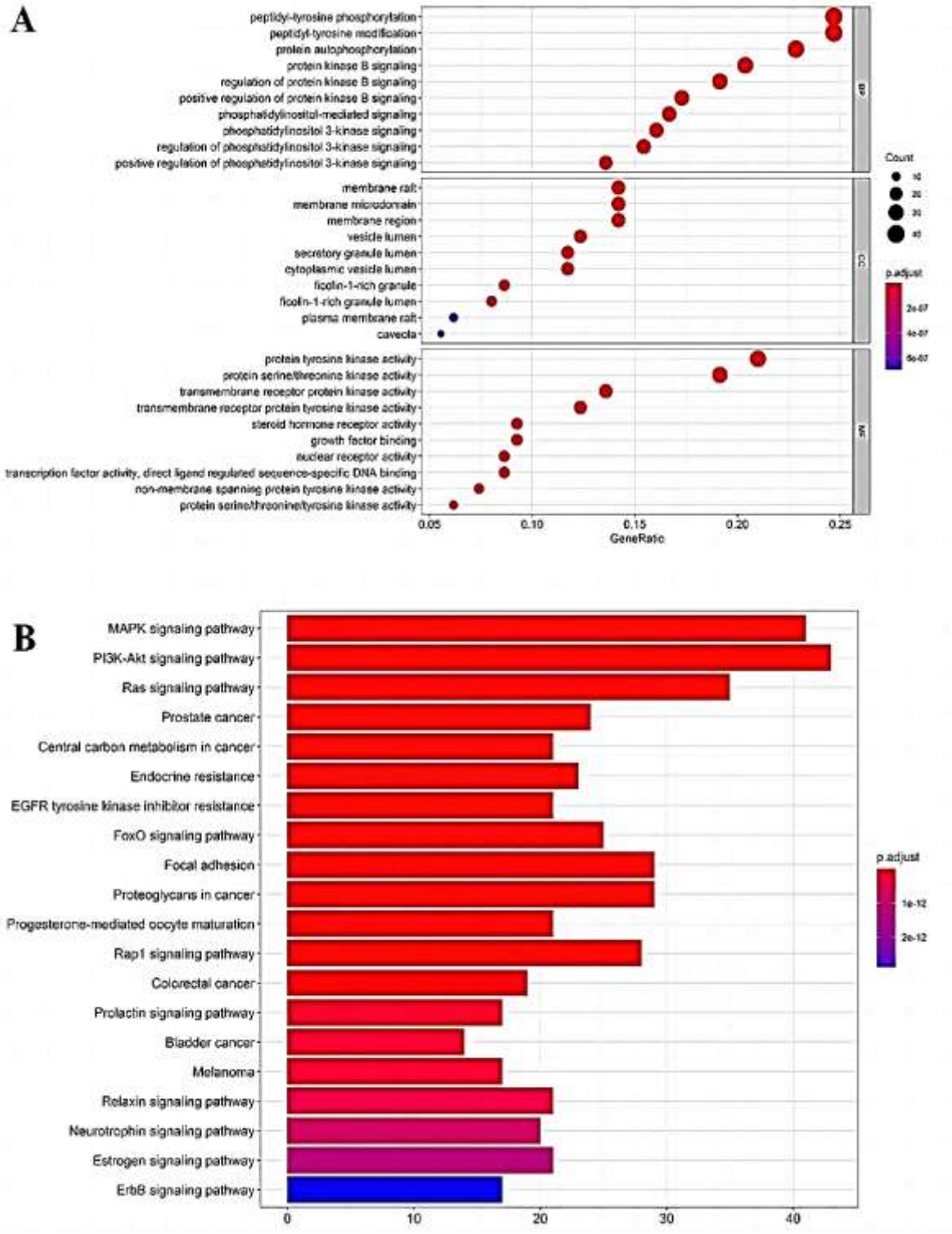

Figure 3: Top 10 and 20 enrichment scores in GO enrichment analysis (A) and KEGG pathway (B)

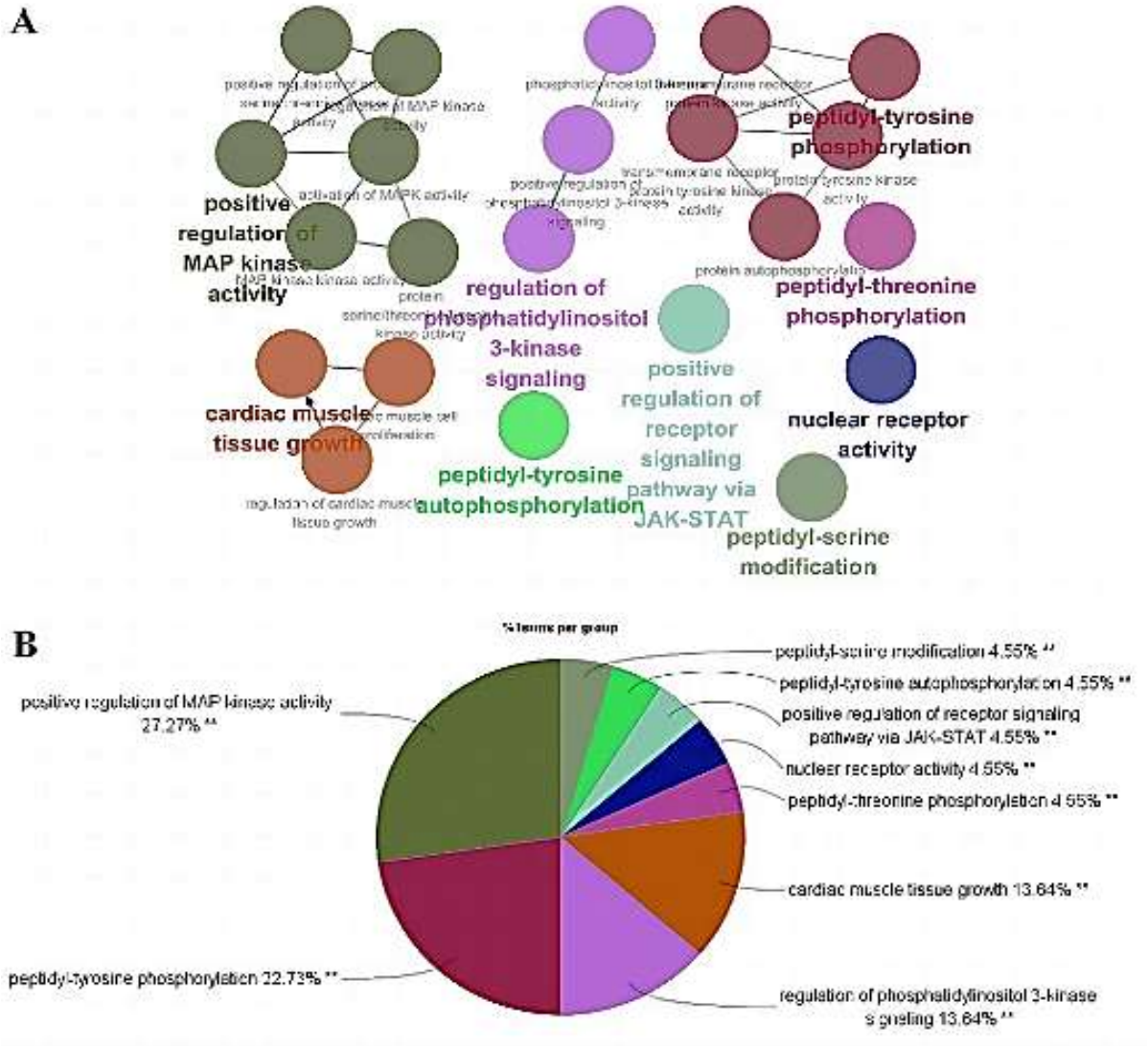

Figure 4: Biological processes enrichment analysis based on ClueGo. (A) Candidate targets for lenvatinib against HCC enriched in the representative biological process, (B) pie-chart from ClueGo

Table 2: Docking parameters and scores

Gene	Protein	ID of PDB	Score (kcal/mol)
HRAS	HRas proto-oncogene, GTPase	121p	-24.2911
BRAF	B-Raf proto-oncogene, serine/threonine kinase	1uwh	-23.9298
TYMS	thymidylate synthetase	1hvy	-23.8444
AKT1	Protein Kinase B 1	1h10	-18.7491
EGFR	Epidermal growth factor receptor	1m17	-15.9306

Impact of lenvatinib on proliferation of HuH-7 Cells

As shown in Figure 6 A, cell survival was lower for lenvatinib treatment at concentrations ranging from 3 to 121.5 μM than for controls at all incubation periods ($p < 0.05$). The differences in the cell survival rates were not statistically significant between 24 and 48 h culture periods when lenvatinib concentration ranged from 9 to 27 μM ($p > 0.05$). However, a significant difference was seen between 24 and 72 h ($p < 0.05$). Compared to the concentration range of 54 to 121.5 μM ($p < 0.05$), when the cell concentration was 9 μM, there were no marked differences in cell survival between periods. In the CCK-8 assay, lenvatinib concentrations of 1, 3, and 9 μM were used for 72 h.

mRNA expression levels

In line with network pharmacology and bound docking analysis, it was confirmed that five core targets of Lenvatinib (AKT1, HRAS, TYMS,

BRAF, and EGFR) bore a close relationship with HCC. Results from qRT-PCR (chart B) showed that, compared with the control group, the large dose (9 μM) group had significantly enhanced mRNA expression level of EGFR, while the mRNA expressions of AKT1, HRAS, TYMS, and BRAF were markedly decreased in the medium dose (3 μM) and large dose (9 μM) groups ($p < 0.01$).

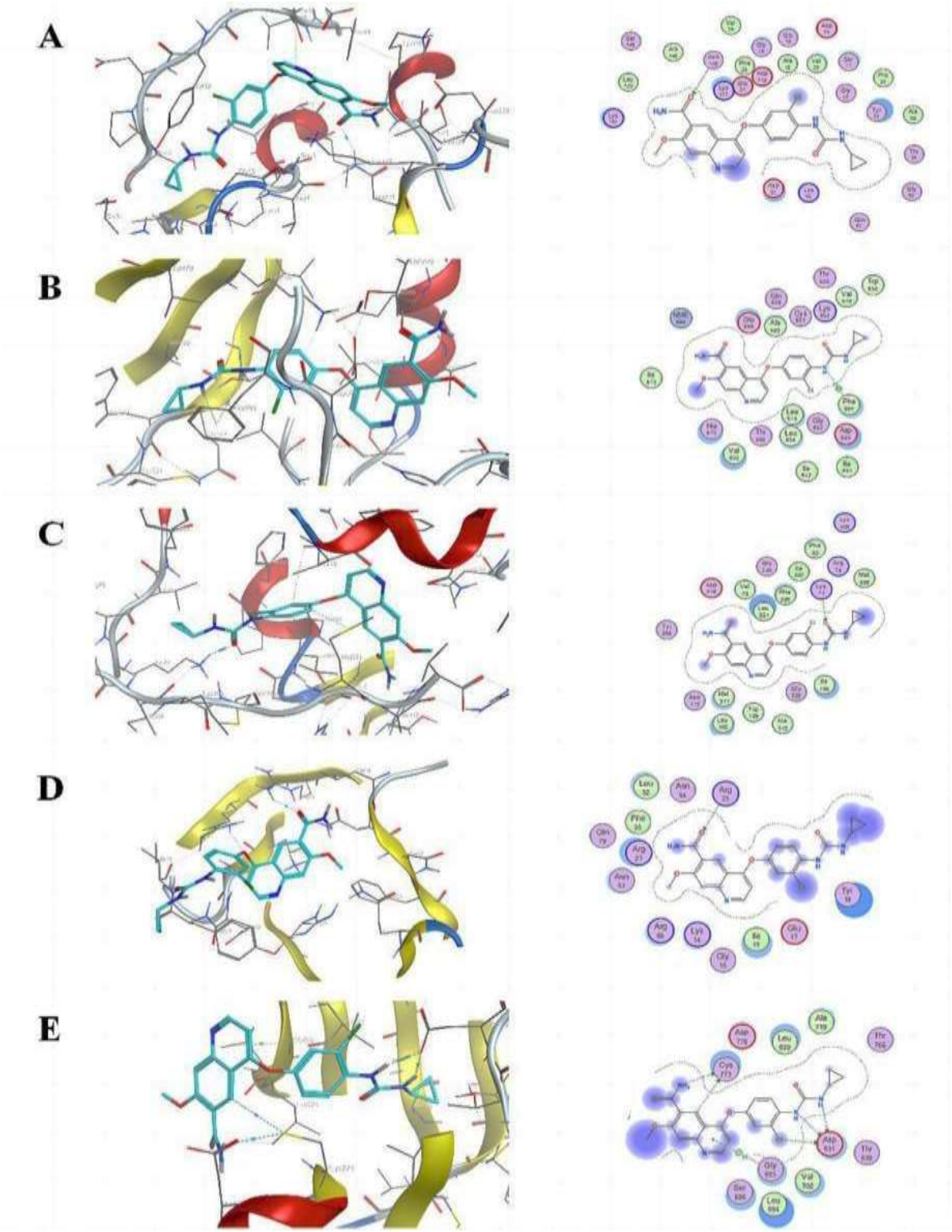

Figure 5: Lenvatinib-target protein binding patterns. (A) HRAS, (B) BRAF, (C) TYMS, (D) AKT1 and (E) EGFR

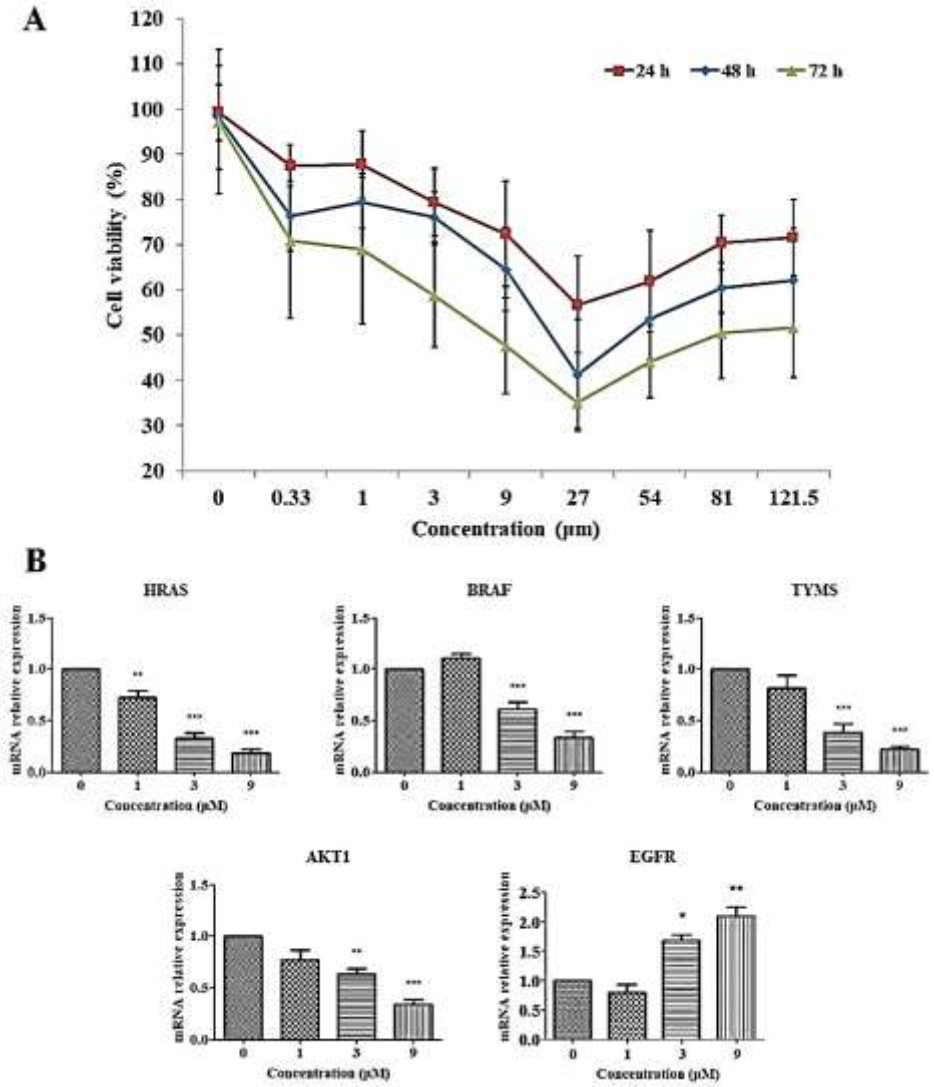

Figure 6: Validation of *in vitro* experiments. (A) Effect of lenvatinib on the viability of HuH-7 cells (n=6) and (B) gene expression levels (n=9). ***P* < 0.05, and ****p* < 0.01, compared with control group

DISCUSSION

In the past, experimentation was the only way to discover the potential activity of a drug. However, with advancements in molecular biology and pharmacology, it is now possible to establish the relationship between drugs and key molecular targets of human diseases using computer technology [12]. The identification and validation of targets is the first step in modern drug development and is one of the bottlenecks in the drug-making process. The identification of potential targets for drug action is also of great importance for safety evaluation of novel drug molecules and elucidation of their mechanisms of action [13]. Network pharmacology is used to expound on the mechanisms underlying drug effects. It creates a new perspective for the analysis of drug effects, and it is currently one of the most important vehicles for elucidation of mechanisms of effects of drugs, as well as the development of new drugs [14]. In the present study, biological information from drug and disease databases was used to predict the interactions between Lenvatinib and potential protein targets in HCC. Thus, the biological function and mechanism of action of Lenvatinib in HCC were determined.

Results from KEGG pathway annotation indicated that the key targets of lenvatinib in its effect against HCC were focused on the MAPK signal pathway, PI3K-Akt signal pathway, Ras signal route, and central carbon metabolism in cancer. These results suggest that lenvatinib may act against HCC *via* multiple targets and pathways, indicating a coordinated network of action. The *p*-value of MAPK signaling pathway was the smallest, suggesting that lenvatinib may exert its anti-tumor effects through the regulation

of this pathway. The MAPK signaling pathway transmits extracellular signals to the nucleus through protein kinases, thereby regulating cell growth, differentiation, apoptosis, and other biological processes [15,16]. The MAPK signaling pathway accounted for 41 of the 154 gene targets, and it was mapped to important node proteins such as Akt1, HRAS, TYMS, BRAF, and EGFR, based on the degree of target association.

In addition, the results of molecular docking showed that lenvatinib was bound most strongly to AKT1, HRAS, BRAF, TYMS, MAP2K1, and EGFR. In particular, AKT which is also referred to as protein kinase B (PKB), after activation by phosphorylation to p-AKT), constitutes an important part of the PI3K-AKT signal pathway which regulates tumor cell proliferation, metastasis, differentiation, and apoptosis [17]. The BRAF gene belongs to the RAF family, and it is one of the most important activators of MEK/ERK, which influence tumor progression by promoting cell proliferation and invasion, among others [18]. Epidermal growth factor receptor (EGFR) is an important regulatory gene that binds to ligands and activates tyrosine kinases through phosphorylation of receptor tyrosine residues, thereby activating important downstream signal routes involved in cell proliferation, migration and apoptosis [19]. It has been reported that EGFR is closely related to tumorigenesis [20]. The thymidylate synthase (TYMS) gene acts as a key target in primary liver cancer in synergy with EGFR [21].

In this study, lenvatinib was chosen as a drug model for investigating the molecular mechanism involved in the anti-HCC effect of lenvatinib through the techniques of network pharmacology, molecular docking, and *in vitro* cellular assays. Nonetheless, this study has some limitations. Firstly, this study used network pharmacology method to identify the potential targets of lenvatinib in its anti-tumor effect on HCC and to validate the key targets through *in vitro* experiments on cell lines. However, *in vivo,* experiments are also necessary. Secondly, the information on the likely targets and PPI covered may not be comprehensive due to complexities in the construction of complete databases. These deficiencies will be rectified in the future with ongoing data updates.

CONCLUSION

This research has predicted five potential therapeutic targets involved in the anti-HCC effect of lenvatinib and has validated the predictions through bioinformatics and *in vitro* experiments. At the same time, KEGG analysis has demonstrated multi-target synergy and found that these targets act through the MAPK signal pathway, PI3K/Akt signal route, Ras signal pathway, and central carbon metabolism in cancer signal pathway. These findings provide a good theoretical basis for development of lenvatinib as an effective anti-HCC drug. Despite achieving the intended research objectives in this study, the current constructed database is still incomplete and requires further optimization. While this trial protocol has demonstrated the feasibility of the approach using the network for predictive analysis, and preliminary validation for identifying potential drug therapeutic targets and key signaling pathways, there is a need for further *in vivo* experiments and clinical validation.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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