

Exploring the Mechanism of *Inula japonica* Thunb. against Non-small Cell Lung Cancer using a computer-aided drug design approach

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Abstract

Context: *Inula japonica* Thunb. (IJT) is widespread to treat non-small cell lung cancer (NSCLC) in China with antiasthma, antitussive, and expectorant effect. However, due to the complexity of compounds and targets, the pharmacological mechanisms of IJT needs further research. The study explores the mechanisms of IJT against NSCLC through network pharmacology, molecular docking and molecular dynamics (MD) simulation. The results showed that quercetin and luteolin were selected as major compounds, and 23 putative targets of IJT against NSCLC were picked out as major hubs. The major targets just modulated the NSCLC pathway, which included Ras, ERBB, MAPK, PI3K-Akt, calcium, and p53 signaling sub-pathways. Moreover, they involved in apoptosis, cell cycle, tumor progression, proliferation, and many other significant biological processes. The molecular docking simulation showed that all the major compounds with NSCLC pathway-relevant targets of IJT had effective binding. Further, MD simulations revealed that the luteolin-AKT1 and quercetin-AKT1 complexes possessed a steady state and bound extremely stably during molecular docking.

Methods: In the present study, the ingredients and targets prediction, compound-target (C-T), and protein-protein interaction (PPI) networks analysis, molecular docking, and MD simulations were applied to mine the anti-NSCLC mechanisms of IJT.

Introduction

Lung cancer is a leading cause of cancer mortality around the world, and associates with poor prognosis. Lung cancer can basically spill into small cell cancer and non-small cell lung cancer (NSCLC). Notably, NSCLC accounts for approximately 85%–90% of lung cancers [3]. The well-recognized pathogenic factors of NSCLC are tobacco consumption, air pollution, second-hand smoke exposure, occupational and environmental exposures, and genetic characteristics [4–6]. The most frequent clinical manifestations of NSCLC include chronic cough, bloody phlegm, chest pain, breathlessness, weight loss, metastasis pain, fatigue, fever, and respiratory distress [7], which seriously have impacts on human health, even life. In addition, most patients with terminal stage NSCLC are not candidates for surgery due to delayed diagnosis. Therefore, patients with advanced NSCLC have to treat with chemotherapy, radiation, or targeted agents to bring their symptoms into remission and give them a glimmer of hope [8, 9]. The therapeutic strategies, while effective to some extent, may also trigger a series of adverse effects, including fatigue, pain, nausea, vomiting, renal toxicity, reduction of lymphocyte, and myelosuppression [10]. Therefore, searching for new strategies against NSCLC is highly clinical significance.

Traditional Chinese medicine (TCM) in combination with chemotherapy is routinely recommended for patients with NSCLC which acts as therapeutic effect through regulation of multiple compounds, targets, and pathways [11, 12]. In addition, TCM can not only relieve chemotherapy-related side effect but also prolong the survival time of patients [13]. Therefore, TCM provides a promising complementary strategy for patients with NSCLC. *Inula japonica* Thunb. (IJT) is a TCM with anti-asthma, cough suppressant, and expectorant properties [14]. Polysaccharides in IJT are reported to be anti-diabetic compounds with β -cell-

protective and antioxidant stress properties [15]. Sesquiterpenes isolated from IJT display significantly inhibitory outcomes on the nitric oxide production of mouse macrophage, indicating that they have potential anti-inflammatory and anti-tumor activities [16–18]. Furthermore, Japonicone A can suppress the growth of NSCLC cells by modulating mitochondria-mediated pathways [19]. Essential oils extracted from IJT can enhance the sensitivity and absorption of breast cancer cells by regulating the relevant mechanism of ABCB1 expression, which eventually exhibit the reversal of multi-drug resistance [20]. Although the anti-tumor activity of IJT has been investigated, the mechanism of IJT against NSCLC is unclear.

The traditional approaches to the study of herbal medicine have focused on the one-compound, one-target, and one-pathway in the treatment of disease, and the complex mechanisms of herbal medicine are difficult to explicit. Fortunately, network pharmacology, a recent emerging technology based on the principles of network theory and systems biology, can be utilized to probe the comprehensive mechanism of drug action [21, 22]. In addition, network pharmacology can clarify and visualize the compound-target and target-disease networks. It can then also be used to systematically elaborate on the potential mechanisms of a drug in the treatment of diseases through network analysis [23]. Therefore, the present study applied network pharmacology, molecular docking and MD simulations to predict the mechanism of IJT against NSCLC. A detailed flowchart is shown in Fig. 1.

Materials and Methods

Collection of candidate compounds from IJT

All compounds of IJT were acquired from three online databases, namely, Traditional Chinese Medicine Systems Pharmacology Database (TCMSP), Traditional Chinese Medicine-Mesh (TCM-Mesh), and The Encyclopedia of Traditional Chinese Medicine (ETCM). Oral bioavailability (OB) and drug-likeness (DL) were employed to screen the potential drug candidate compounds. The thresholds of OB and DL were set greater than or equal to 30% and 0.18, respectively. Ingredients of IJT, which satisfied the above criteria, would be candidate drugs. The simplified molecular input line entry specification (SMILES) of candidate compounds was acquired from the PubChem database for target prediction.

Collection of NSCLC-relevant targets of IJT

The putative compound targets were obtained from Swiss Target Prediction and TCMSP databases. In the Swiss Target Prediction database, the SMILES format of each candidate compound was imported, and species was set to "*Homo sapiens*". The probability of putative targets was set more than 0.50 for the sake of the credibility of targets. In the TCMSP database, the potential targets for every candidate compound were also collected as supplement. We took "non-small cell lung cancer" as the keyword to search for disease targets in two separate databases. One was the Therapeutic Target Database, which could provide information of known and grouped therapeutic targets. The other was the DisGeNET database, which embodied genes and variants linked to human diseases. In the end, the compound

targets and disease targets were obtained by consolidating the two data and removing duplicates, respectively.

The different target names were transformed into gene symbols by the UniProt database. We mapped ultimately compound targets into NSCLC-relevant targets via the OmicShare platform. The common targets were the targets of IJT acting on NSCLC.

Collection of protein-protein interactions (PPIs)

Protein-protein interactions (PPIs) were acquired from High-quality INTERactomes (HINT) and STRING databases. HINT, which gathered eight interactome resources, namely, BioGRID, MINT, iRefWeb, DIP, IntAct, HPRD, MIPS, and PDB, could acquire high-quality PPIs, and be updated every night. The STRING database, which furnished the physical and functional interactions, was acquired from the integration of experimental data, computation-based prediction, and literature mining. Then, we merged PPIs from two databases and deleted redundant entries.

Network construction

Two networks were constructed as follows: 1) the compound-target (C-T) network was established for candidate compounds in IJT and their NSCLC-related targets, and 2) The PPI network was constructed for NSCLC-related targets in IJT and other therapeutic targets of NSCLC.

The two networks were visualized using the Cytoscape software (3.6.0). A network analyzer was used to calculate the topological parameters. Degree, one of the topological parameters, referred to the quantity of linkage to node i . If the degree value of a node was larger, it indicated that the node sat at the heart of the network. Hence, degree value was used to weigh the importance of a node in the network. In general, the node was regarded as a major hub if its degree was twice higher than the average degree value of all other nodes.

Enrichment analysis

To further clarify the biological functions and significant pathways of NSCLC-related targets in IJT, we applied the DAVID Bioinformatics Resources (version 6.8) to perform Gene Ontology (GO) and KEGG pathway enrichment analysis. In general, GO terms and KEGG pathways with P values less than 0.05 were considered significant.

Molecular docking

To verify the binding affinity of core targets with significant compounds in IJT, we used the Autodock vina to implement molecular docking. First, the 3D structures of significant compounds in IJT were directly acquired from the PubChem database, and the crystal structures of the major targets of NSCLC-related IJT were downloaded from the PDB database. Then, the protein preparation protocol was used before molecular docking, which included removal of unwanted chains, ligands, water, and metal ions, addition

of hydrogenation, calculation of point charges, and insertion of missing atoms in incomplete residues using the Autodock tool. Every protein was considered as a receptor. The protein active site of the receptor could be found as a cavity. Grid box included the active site of the target protein, and was eventually obtained. In addition, compounds also required pre-processing, such as calculating the charge, setting the atomic type and number of rotating bonds, etc. Then, the modified compounds and target proteins were imported into Autodock vina for molecular docking. Binding energy was ultimately obtained to assess the effects of molecular docking. In general, if binding energy was lower, it indicated that docking conformation was more stable and reliable.

Molecular dynamics simulation

MD can simulate the molecular motion trajectory of molecules in the organism environment by computer. On the basis of the molecule-protein complexes obtained from the foregoing docking as initial structures, all-atom molecular dynamics simulations were performed using Gromacs (2021.4) to validate the experimental results, respectively. We opted for CHARMM 36 as the force field to determine the stability of the ligand within the binding site, and employed the TIP3P water model. Sobtop was utilized to optimize the charge of the ligand and generated the files required for the molecular dynamics parameterization[24]. The boundaries of the box have a minimum distance of 1 nm from the atoms of the protein-ligand complex. We added 0.145 mol/L of neutral sodium chloride solution in order to simulate the human environment. The physical conditions were set as constant temperature (310K) and constant pressure (101kPa). Following the energy minimization, NVT and NPT equilibration, the state of all environments was then at equilibrium at the set temperature and pressure. The system was eventually subjected to 50 ns of molecular dynamics simulations, in which the conformation was stored every 10 ps. The results of the MD simulations were analyzed and visualized by root mean square deviation (RMSD) and radius of gyration (Rg) using the Gromacs embedded program and Origin. Generally, root mean square deviation (RMSD) was considered as a measure of system stability, and the smaller the average RMSD value, the more stable the system. The Rg of a protein reflected the volume and structural state of the protein. The larger the Rg value of the same system, the greater the expansion of the system that occurs during MD, and the less stable the system.

Results

Candidate compounds of IJT and its corresponding targets

A total of 69 compounds in IJT were acquired from TCMSP, TCM-MESH, and ETCM databases after removing duplicates. 20 compounds of IJT accorded with the criteria of OB and DL at > 30% and > 0.18. Since 4 compounds had no corresponding targets, they were consequently disregarded. As a result, 16 candidate compounds, including 8 flavonoids, 1 triterpenoid, 4 sesquiterpenoids, 2 phytosterols, and 1 organic acid, were acquired, and hit 280 targets altogether via the target prediction systems of TCMSP and Swiss Target Prediction databases. The detailed information of the candidate compounds in IJT and its corresponding putative was listed in Table 1 and **Table S1**, respectively.

Table 1
Detailed information about candidate compounds of IJT

Compound Name	PubchemID	Categories	OB%	DL
inulicin	N/A	sesquiterpenoid	30.12	0.22
Tamarixetin	5281699	flavonoid	32.86	0.31
Britanin	5315501	sesquiterpenoid	33.73	0.41
Chryseriol	5280666	flavonoid	35.85	0.27
luteolin	5280759	flavonoid	36.16	0.25
Melilotoside	5280759	organic acid	36.85	0.26
beta-sitosterol	222284	phytosterol	36.91	0.75
1,6-O,O-diacetylbritannilactone	10360513	sesquiterpenoid	39.03	0.31
Pratensein	5281803	flavonoid	39.06	0.28
Isoramanone	50716134	phytosterol	39.97	0.51
kaempferol	5280863	flavonoid	41.88	0.24
Taraxasterol acetate	13889352	triterpenoid	43.08	0.74
quercetin	5280343	flavonoid	46.43	0.28
Patuletin	5281678	flavonoid	53.11	0.34
Azaleatin	5281604	flavonoid	54.28	0.3
AC1L9CIK	442263	sesquiterpenoid	73.35	0.22

NSCLC-related targets of IJT

The quantity of NSCLC-related therapeutic targets from the TTD and DisGeNET databases was 63 and 65, respectively. A total of 110 NSCLC-related targets were ultimately obtained by removing the duplicates. The details of NSCLC-relevant targets were depicted in Table S2. In addition, 24 NSCLC-related targets of IJT were acquired by overlapping dramatically of compound targets and NSCLC-relevant targets (**Table S3** and Fig. 2), which might be the potential therapeutic targets of IJT against NSCLC.

C-T network

TCM exerted extensive biological and pharmacological activities via multi-compound and multi-target. Hence, we built the C-T network to explore the sophisticated interactions based on the compounds and their related targets on NSCLC at a systems level. As displayed in Fig. 3, the C-T network consisted of 40 nodes (16 compounds and 24 NSCLC-related targets of IJT) and 60 edges. Meanwhile, the mean value of degree was 3.75 (**Table S4**). It signified that per compound correlated with at least 3.75 targets.

Eventually, Quercetin and luteolin with degree > 7.5 became crucial active compounds of IJT in the treatment of NSCLC.

Similarly, the median degree value of targets was 2.5. It meant that per target was modulated by at least 2.5 compounds. Targets with high degree value were PTGS2 (16 interactions), CDK2 (7 interactions), and CHEK1 (4 interactions). From the above analysis, IJT exhibited synergistic or additive effects on NSCLC through multi-compound and multi-target.

PPI network for IJT acting on NSCLC

To clarify the potential mechanisms of IJT acting on NSCLC, 677 protein-protein interactions between the NSCLC-related targets of IJT, known therapeutic target, and interactional human proteins were visualized by Cytoscape software. PPI network embodied 476 nodes, and 677 edges (Fig. 4 and **Table S5**). The degree values of 23 compound targets were twice higher than the average degree (3) of all other nodes, and therefore they were encoded as candidate hub, including cellular tumor antigen p53 (TP53), epidermal growth factor receptor (EGFR), cyclin-dependent kinase inhibitor 2A (CDKN2A), G1/S-specific cyclin-D1 (CCND1), Vascular endothelial growth factor A (VEGFA), Myc proto-oncogene protein (MYC), E3 ubiquitin-protein ligase Mdm2 (MDM2), RAC-alpha serine/threonine-protein kinase (AKT1), and receptor tyrosine-protein kinase erbB-2 (ERBB2), etc. The detailed information is provided in **Table S6**.

GO and KEGG pathway enrichment analysis

The GO and KEGG enrichment analysis was used to comment on the 23 major targets of IJT against NSCLC. GO classified function into categories of biological process (BP), molecular function (MF), and cellular component (CC). We set the level of statistical significance at $P < 0.05$. A total of 72 GO enrichment terms were obtained, including biological process (BP) (47 terms), cellular component (CC) (10 terms), and molecular function (MF) (15 terms). Then, the top 10 significant GO terms in the BP, CC, and MF categories were displayed in Fig. 5. The top 10 terms in the biological process category were transmembrane receptor protein tyrosine kinase signaling pathway, negative regulation of apoptotic process, cell proliferation, protein sumoylation, etc. Cellular components included protein complex, nucleus, receptor complex, and nucleoplasm, etc. In the molecular function category, there were transmembrane receptor protein tyrosine kinase activity, protein tyrosine kinase activity, receptor signaling protein tyrosine kinase activity, and protein kinase activity, etc.

According to the results of the KEGG pathway enrichment analysis, 23 major targets were mapped onto 33 significant pathways ($P < 0.05$, **Table S7**). The bubble chart of the top 10 KEGG pathways according to significance was depicted in Fig. 6. IJT regulated significantly multiple types of cancers pathway, incorporating endometrial cancer, pancreatic cancer, glioma, non-small cell lung cancer, prostate cancer, melanoma, pathways in cancer, proteoglycans in cancer, etc. IJT also modulated cancer-related pathways, such as cell cycle, PI3K-Akt signaling pathway, FoxO signaling pathway, etc. Therefore, the potential mechanisms of IJT in the treatment of NSCLC might be mainly attributed to its synergistic

modulation on the relevant pathways of cancers. Interestingly, “non-small cell lung cancer” had just been included, which enriched TP53, CCND1, ERBB2, CDKN2A, EGFR, ALK, and AKT1. “non-small cell lung cancer” was considered the significant pathway of IJT acting on NSCLC.

Molecular docking validation

The two compounds (quercetin and luteolin) as ligands, and NSCLC pathway-modulated targets (TP53, CCND1, ERBB2, CDKN2A, EGFR, ALK, and AKT1) as receptors were validated by molecular docking. Binding energy was used to measure the docking effect of compound-target. In general, the binding energy less than -6.0 kcal/mol indicated a certain binding activity between the ligand molecule and the receptor protein, and binding energy less than -7.0 kcal/mol indicated a good binding activity, while binding energy less than -8.0 kcal/mol implied that the active compound with target had strong stability. The results of molecular docking showed that all compound-target exhibited effective docking. The detailed information was described in **Table S8**. The result of molecular docking was simultaneously drawn heat map in Fig. 7. It was noted that the binding energy of 8 pair compound-target (57%) was less than -7 kcal/mol, indicating that they had good binding activity. Especially, luteolin with AKT1 (-9.5 kcal/mol), quercetin with AKT1 (-9.4 kcal/mol), quercetin with EGFR (-7.8 kcal/mol), and luteolin with ALK (-7.7 kcal/mol) owned the lower energy binding and more stable conformation (Fig. 8).

MD simulations

The most tightly bound combinations of luteolin-AKT1 and quercetin-AKT1 in molecular docking were selected for molecular dynamics simulations. To evaluate the structural stability of protein-ligand complexes and the tertiary structural stability of protein-ligand binding during MD simulations, RMSD values and Rg values were calculated for the complexes. From the graph of the RMSD results of MD simulations (Fig. 9A), it could be noted that the RMSD values of the luteolin-AKT1 complex and quercetin-AKT1 complex fluctuated considerably during the initial phase of the simulation ($0 \sim 20$ ns), which indicated that there were definite changes in the conformations of the two complexes in the molecular dynamics simulation. After 20 ns, the RMSD values of luteolin-AKT1 complex primarily fluctuated between $0.7589151 \sim 0.8879525$ nm, and the RMSD values of quercetin-AKT1 complex largely ranged from $0.3397707 \sim 0.6519626$ nm. The RMSD fluctuations were within a reasonable range, which indicated that the luteolin-AKT1 and quercetin-AKT1 complexes were in steady state and the bindings were extremely stable. As seen in the graph of Rg results from MD simulations (Fig. 9B), the magnitude of fluctuations (extent of fluctuations) and RMSD of the two complexes were consistent. In terms of RMSD and Rg, luteolin-AKT1 (black dash) showed a superior protein hydrophobicity and a more stable tertiary structure than the molecular dynamics system formed by quercetin-AKT1 (red dash). However, both two complexes had excellent properties and could exist stably without affecting their own and other protein structures. However, both two complexes have excellent properties and could exist stably without affecting their own and other protein structures. Therefore, it was concluded that luteolin and quercetin acting on AKT1 could be therapeutically useful for improving and treating NSCLC.

Discussion

IJT was commonly used as an adjunct drug in the treatment of NSCLC for clearing away the lung heat, eliminating phlegm, and relieving cough in the folk. In the study, network pharmacology was applied to dissect underlying mechanisms of ILT in treating NSCLC from holistic perspective.

C-T network disclosed that quercetin and luteolin were considered to play a critical role in treating NSCLC. It was reported that quercetin exhibited extremely antiproliferative, proapoptotic, and antimetastatic activities on NSCLC by enhancing cytotoxic efficacy [25], regulating the pathways related to the inhibition of snail-dependent Akt activation and Snail-independent ADAM9 expression [26], mediating BCL2/BAX gene [27], and regulating-down IL-6/STAT-3 signals [28]. Similarly, luteolin possessed wide spectra of pharmacological actions, including anti-inflammation [29], neuroprotective effect [30], protection against nonalcoholic steatohepatitis [31], anti-tumor [32], anti-ischemia injury [33], and anti-diabetic cardiomyopathy [34]. A recent pharmacological study presented that luteolin could modulate the expression of genes relevant to steroidogenesis, apoptosis, and stress response, such as Fas, Tp53, and PARP. Furthermore, luteolin contributed to the treatment of NSCLC by decreasing AIM2 mediation [35]. Therefore, quercetin, and luteolin exerted significantly therapeutic effect on NSCLC, and then might be main elements of IJT in the treatment of NSCLC. In addition, prostaglandin G/H synthase 2 (PTGS2, COX2) and cyclin-dependent kinase 2 (CDK2) targeted 16 and 7 compounds, which played significant pathological roles in inflammation, cancer, and immunoreactivity [36–38]. For example, the overexpression of COX2 could lead to various cancers, such as colorectal, gall bladder, gastric, prostate, breast, lung, endometrial, and skin cancers. So COX2 was recognized to play a significant role in the pathophysiological processes of tumor growth [39–41]. Proliferation, migration, and invasion of prostate cancer cells were regulated by toll-like receptor 4/cyclooxygenase-2 (COX2) by activating NF- κ B [41]. In addition, the level of COX2 was reduced by targeting the PGE₂-mediated activation of β -catenin signaling. As a result, the migration of NSCLC cells was inhibited [42]. CDK2 was correlated with the poor outcome of NSCLC, which indicated that CDK2 might be the potential target against NSCLC. To sum up, components and targets of IJT exhibited synergistic or additive effects on NSCLC.

Conclusion

Network pharmacology, molecular docking, and MD simulations technology were applied to predict the mechanism of IJT on NSCLC. The findings provided new insights into the mechanisms of IJT in the treatment of NSCLC. Nonetheless, further experimental validations of the prediction results are required in our future studies.

Declarations

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Availability of data and materials

The data used to support the findings of this study are all included within the article.

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Author contribution

Huiqin Qian and Bailing Wang conceived and designed the research methods. Huiqin Qian wrote the paper.

Ethical approval

Not applicable.

Competing interests

All the authors declare no conflict of interest.

Statement

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Supplementary materials

Table S1 Putative targets in IJT

Table S2 Known therapeutic NSCLC-related targets

Table S3 Known therapeutic targets of IJT acting on NSCLC

Table S4 Topological features of all nodes in the compound-compound target network

Table S5 Protein-protein interactions of therapeutic targets of IJT on NSCLC

Table S6 Topological features of all nodes in the T-T network of putative IJT targets, known therapeutic target and interactional human proteins

Table S7 Significant pathways

Table S8 The results of molecular docking

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Figures

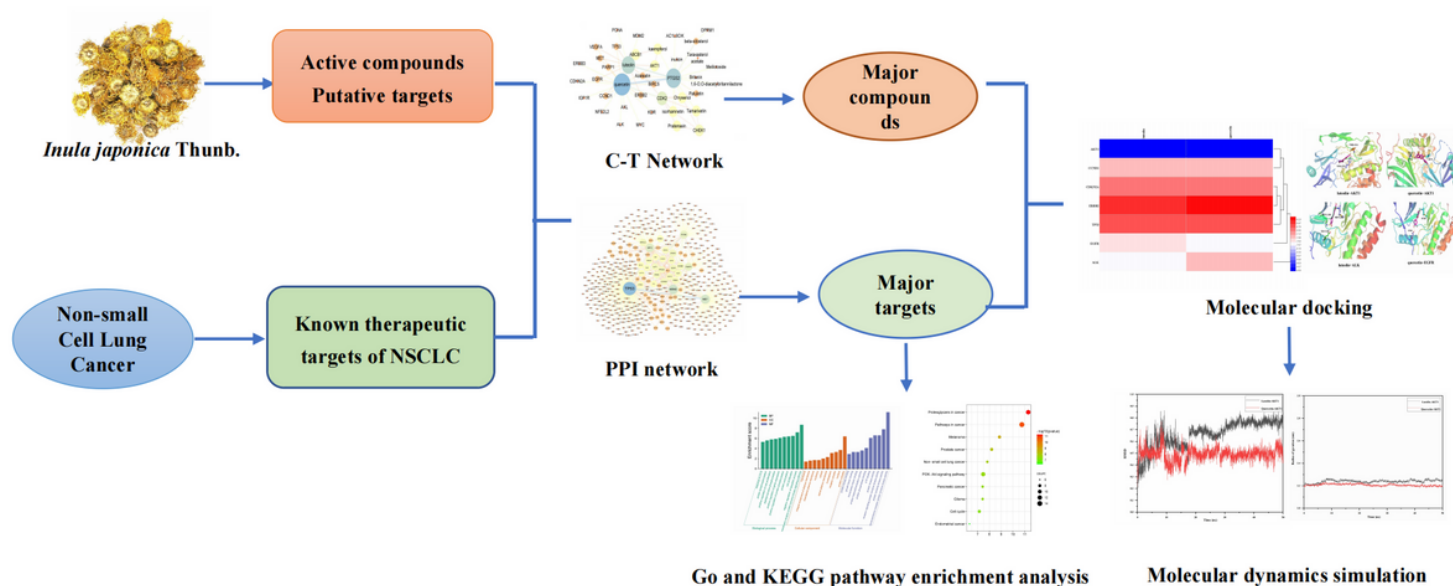


Figure 1

Workflow of the study

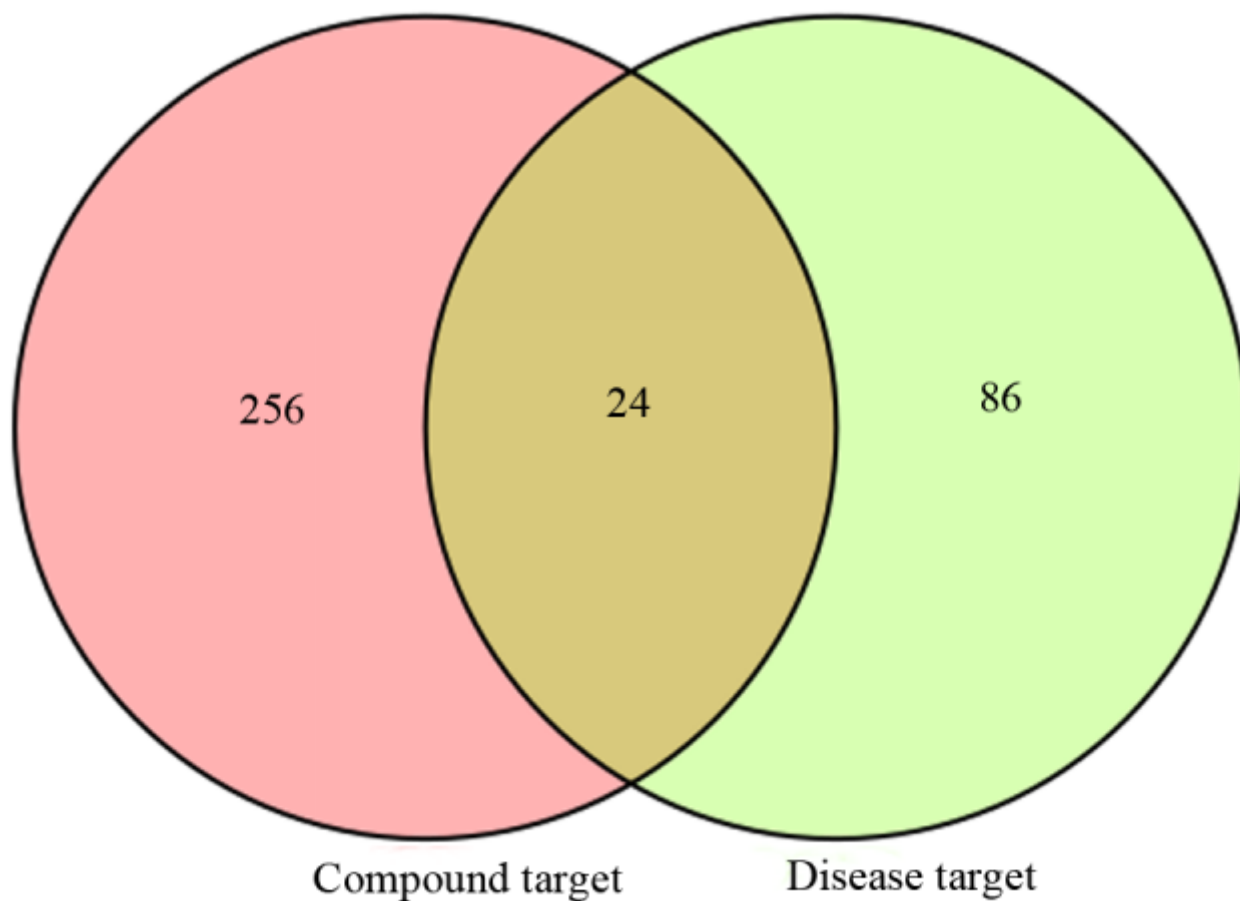


Figure 2

The Venn chart

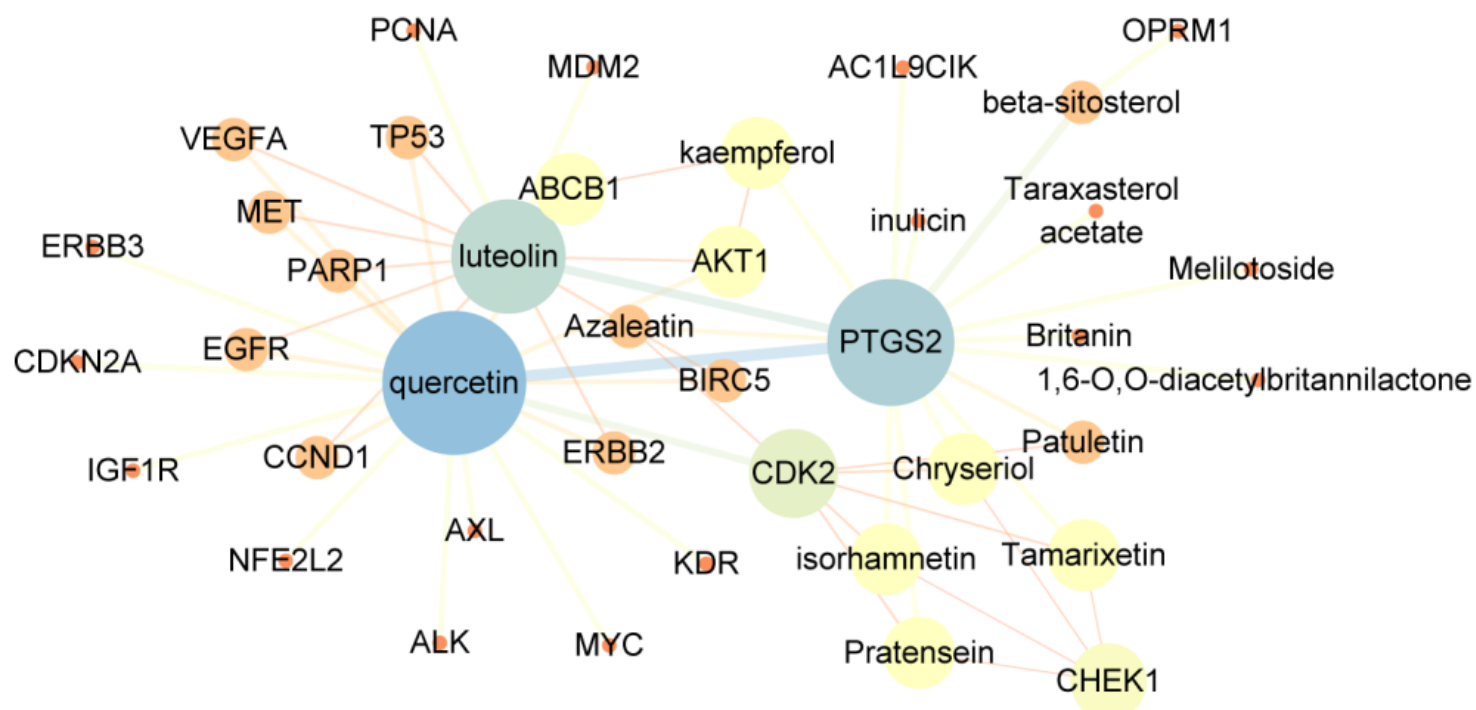


Figure 3

Compound-target network

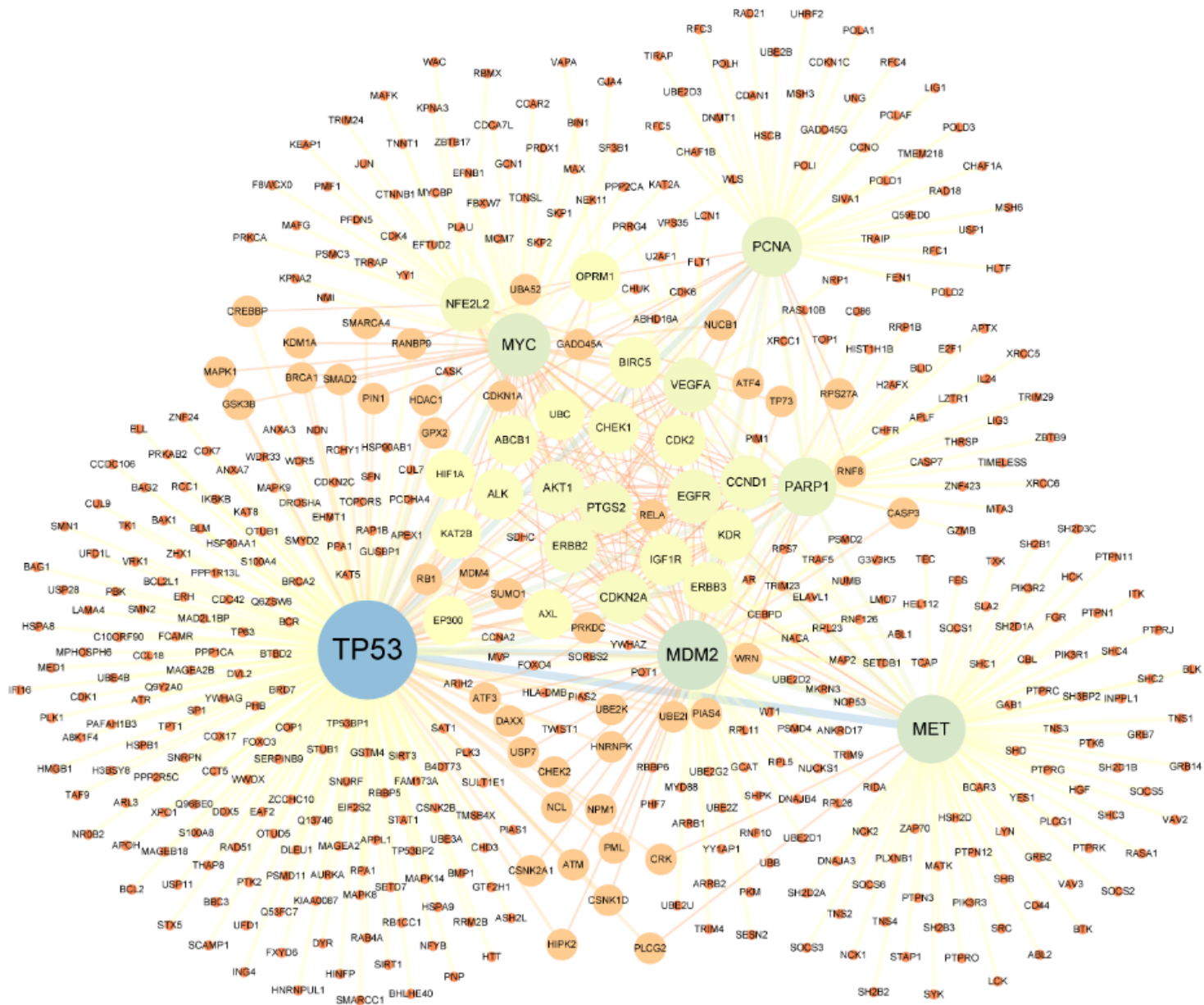


Figure 4

PPI Network

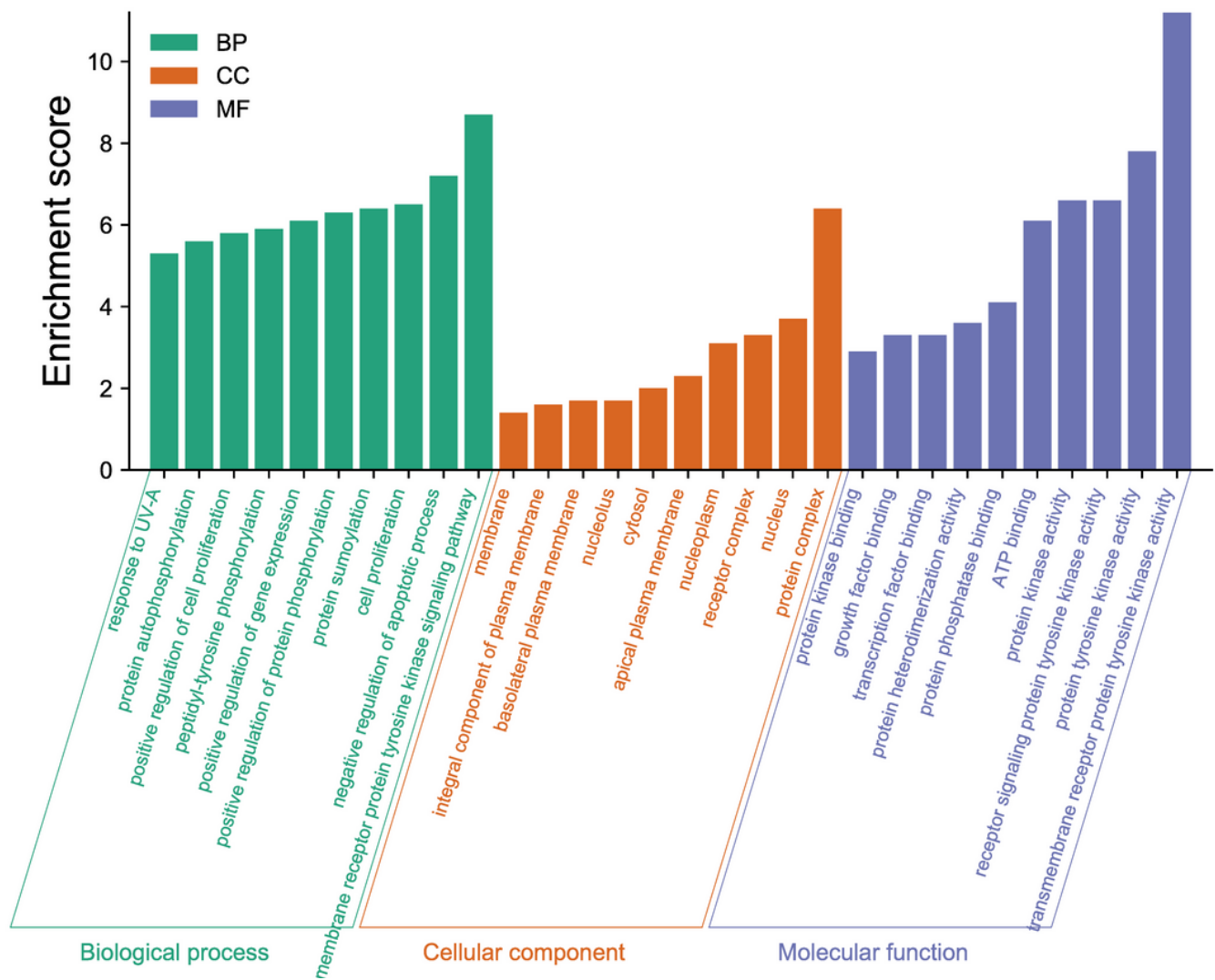


Figure 5

The top 10 significantly enriched terms in BP, CC and MF categories

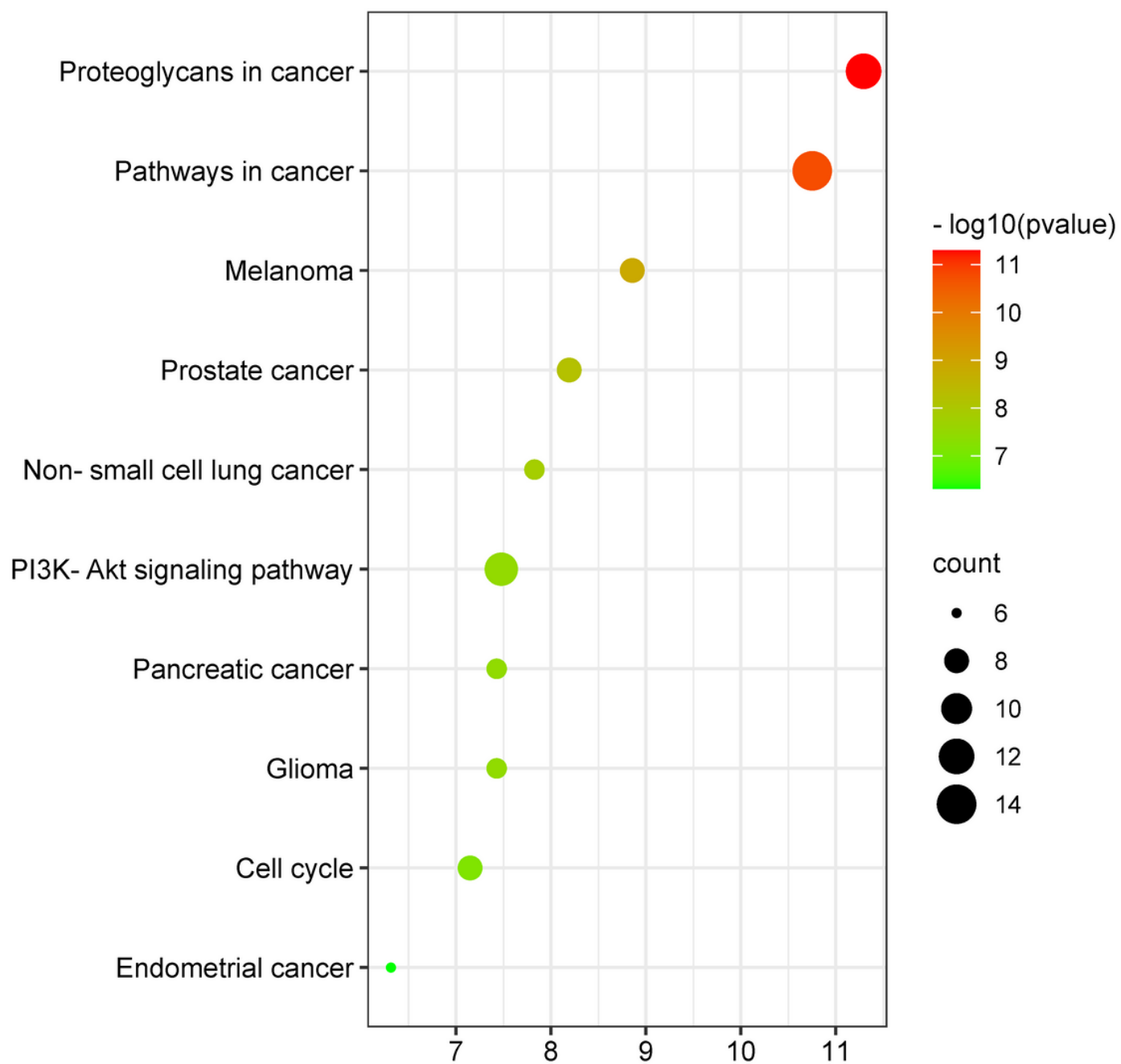


Figure 6

KEGG pathway enrichment analysis of the putative targets of IJT against NSCLC

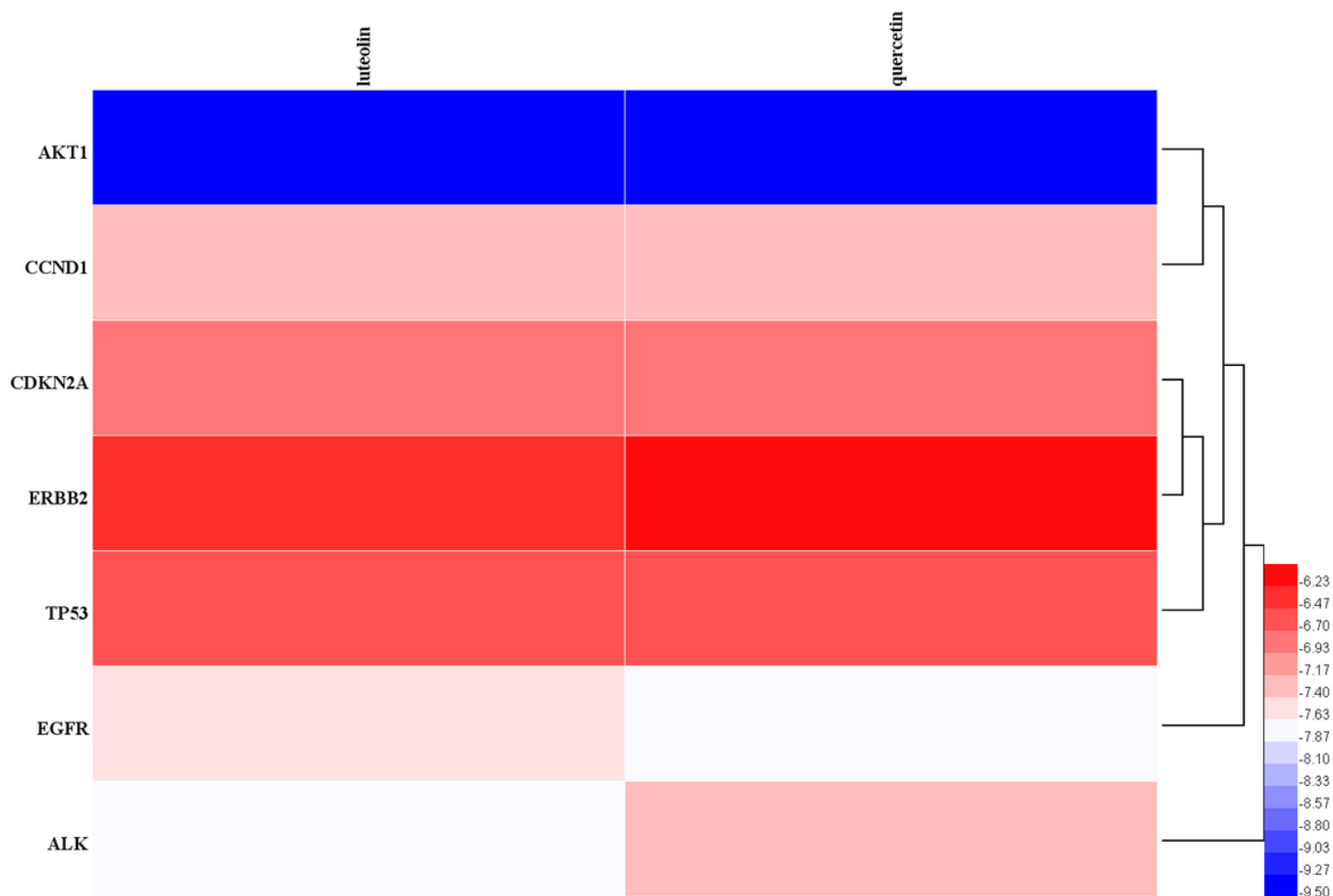


Figure 7

The heat map of molecular docking

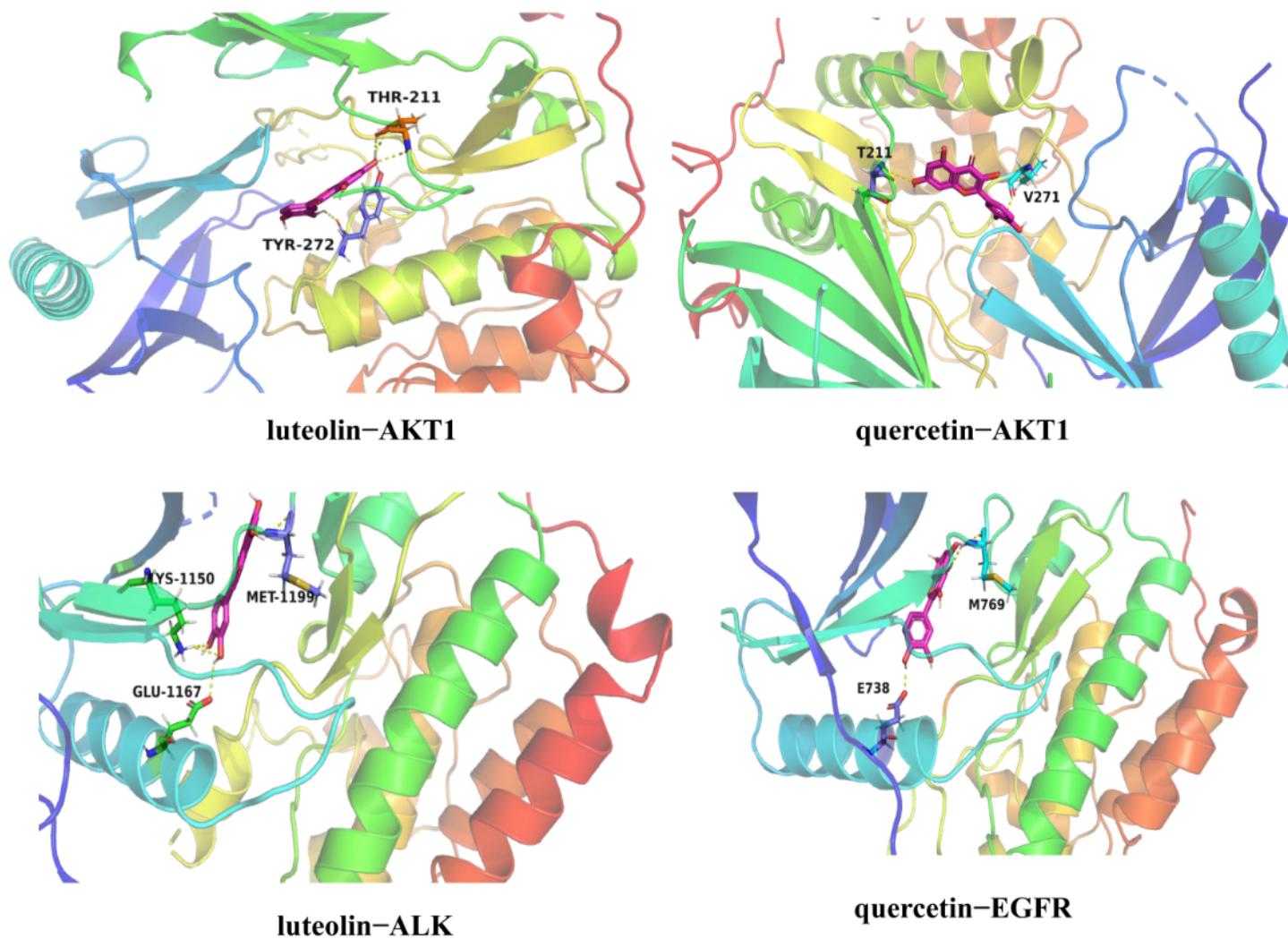


Figure 8

Molecular docking pattern diagram

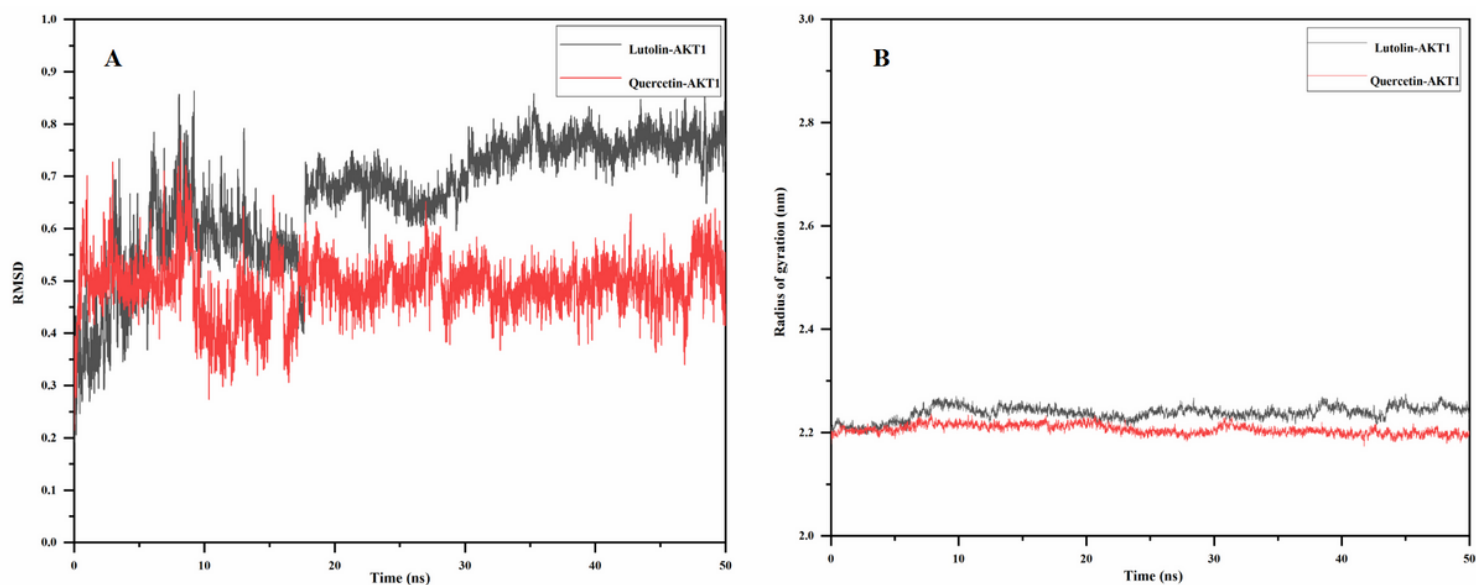


Figure 9

MD simulation. (A) RMSD of the comple. (B) Rg variation diagram of the complex

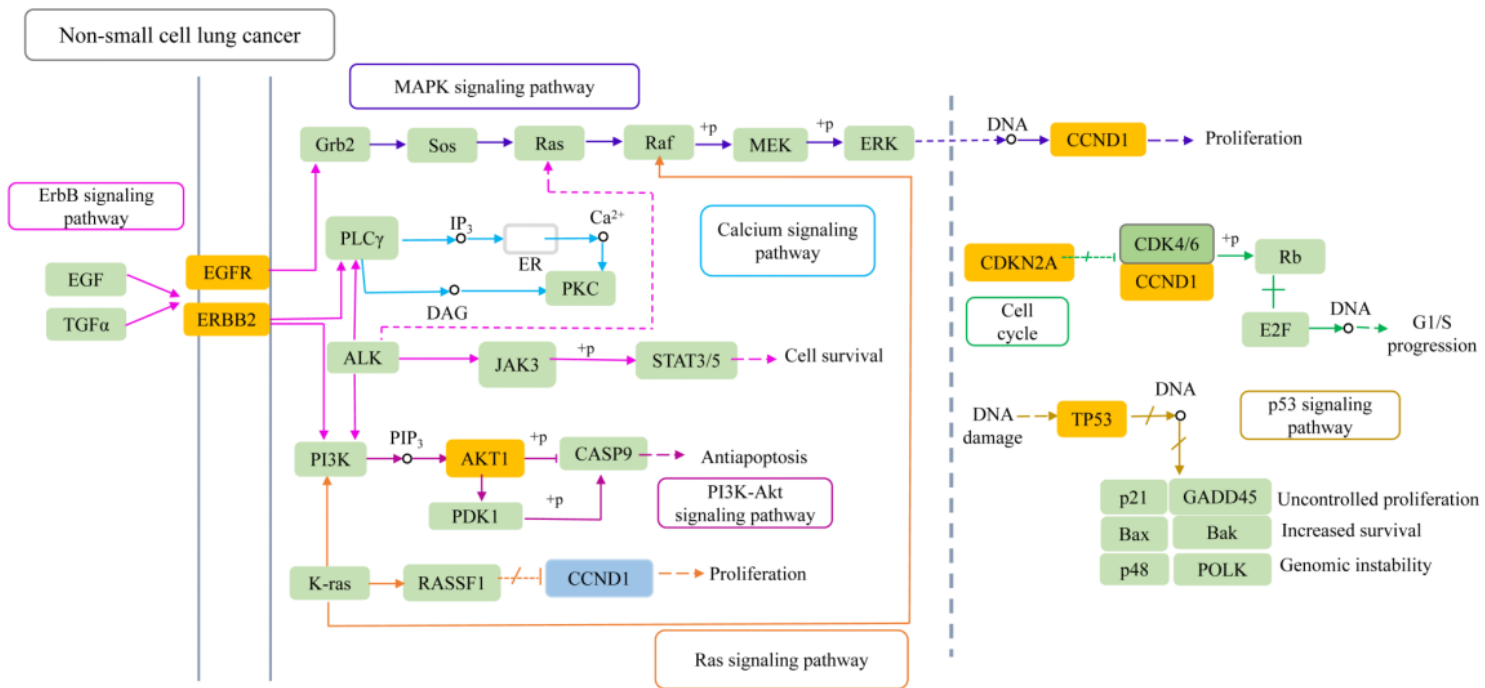


Figure 10

NSCLC-pathway

Supplementary Files

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- [TableS.doc](#)