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Network pharmacology study of *Citrus reticulata* and *Pinellia ternata* in the treatment of non-small cell lung cancer

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ABSTRACT

It has been recognized that *Citrus reticulata* and *Pinellia ternata* have a good therapeutic effect on NSCLC. However, the potential mechanism of *C. reticulata* and *P. ternata* in the treatment of NSCLC based on network pharmacology analysis is not clear. The "Drug-Component-Target-Disease" network was constructed by Cytoscape, and the protein interaction (PPI) network was constructed by STRING. Our study indicated that 18 active ingredients of *C. reticulata* and *P. Ternata* were screened from the TCMSP database, and 56 target genes of *C. reticulata* and *P. Ternata* for the treatment of NSCLC were identified, and we constructed the "Drug-Component-Target-Disease" network. In this study, we screened 56 PPI core genes to establish a PPI network. We concluded that the network pharmacology mechanism of the effect of *C. reticulata* and *P. Ternata* on NSCLC may be closely related to the protein expressed by TP53, ESR1, FOS, NCOA3 and MAPK8, and these may play the therapeutic roles by regulating the IL-17 signaling pathway, antigen processing and presentation, microRNAs in cancer and endocrine resistance.

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Introduction

Lung cancer is the leading cause of cancer-related death in the world, 80% of which are non-small cell lung cancer (NSCLC) (1). In 2020, more than 2 million patients died of lung cancer, which is expected to increase to 3 million by 2035, and would be a major health threat (2, 3). At present, NSCLC is mainly treated by surgery, targeted drug therapy, chemotherapy and radiotherapy (4-6). However, more researches on immunotherapy of immune checkpoint inhibitors (ICIS) have been carried out, which provides a new idea for the treatment of lung cancer (7-9). Additionally, traditional Chinese medicine is particularly important in improving the therapeutic effect of lung cancer, reducing the side effects of radiotherapy and chemotherapy and improving the quality of life (10, 11).

Citrus reticulata and *Pinellia ternata* are commonly used Chinese herbal medicines with the function of regulating qi and resolving phlegm (12-14), which are mostly related to respiratory diseases in clinical treatment. *C. reticulata* and *P. Ternata* are both pungent and warm and belong to the spleen and lung meridians (15-17). Studies have found that phlegm syndrome is the main syndrome of patients with lung cancer, and in recent years, *C. reticulata* and P. *ternata* are the main drugs for the treatment of cancer, especially lung cancer (18-21). Pharmacological studies also showed that Erchen decoction, mainly composed of *C. reticulata* and *P. Ternata*, could reverse the multidrug resistance of tumor cells, and its effect was mainly related to the decreased expression of multidrug resistance-associated protein (MRP)1 and P-glycoprotein (P-gp) through Jun N-terminal kinase (JNK) signal transduction pathway (22, 23). However, due to the Chinese herbal medicine working by regulating multiple proteins, it is still difficult to effectively,

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scientifically and comprehensively explain the mechanism of action of *C. reticulata* and *P. ternata* in the treatment of lung cancer.

Network pharmacology is a drug design method that could be used to study the correlation between active components and target genes of traditional Chinese medicine more efficiently, including system biology, network analysis, connectivity, redundancy and pleiotropy (24-26). It could be used to evaluate the effective targets and pathways of traditional Chinese medicine more scientifically and comprehensively (27, 28). However, the potential mechanism of *C. reticulata* and *P. ternata* in the treatment of lung cancer has not been fully understood yet. Therefore, it is of great significance to study the pharmacological network mechanism of *C. reticulata* and *P. ternata* to clarify the therapeutic mechanism of non-small cell lung cancer. In this study, active ingredients and target genes of *C. reticulata* and *P. ternata* were screened by searching TCMSP (29, 30). The "Drug-Component-Target-Disease" network was constructed by Cytoscape, and the protein interaction (PPI) network was constructed by STRING. R language software was used to classify and enrich the key target genes by Gene Ontology (GO) and the pathway with the Encyclopedia of genomes (KEGG). The results of this study provide a theoretical basis for *C. reticulata* and *P. ternata* in the treatment of non-small cell lung cancer.

Materials and methods

Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP)

The components and target genes of Citrus reticulata and P. ternata were obtained by the TCMSP database (30). According to the principle of pharmacokinetics (ADME) (31), the active components of C. reticulata and P. ternata could be obtained by oral drug similarity (DL) ≥ 0.18 and bioavailability (OB). The target genes corresponding to the active components of *C. reticulata* and *P. ternata* were obtained by Perl language.

Genecards

Genecards is a comprehensive database of human genes, covering several databases for gene analysis (32). "Non-small cell lung cancer (NSCLC)" was used as a keyword to search for NSCLC related genes. The target genes corresponding to the active components of *C. reticulata* and *P. ternata* were mapped to the related genes of non-small cell lung cancer, and the intersection of the two data sets was taken to obtain the gene combination of NSCLC, C. reticulata and *P. ternata*.

Cytoscape

Cytoscape is software that can display the network graphically, also with analyzing and editing (33). It is also a common software to study the network pharmacology of traditional Chinese medicine. In this study, Cytoscape (version 3.6.1) was used to import the data of active ingredients of *C. reticulata* and *P. ternata*, and the target genes of non-small cell lung cancer to construct the "Drug-Component-Target-Disease" network. "Nodes" represent C. reticulata and *P. ternata*, non-small cell lung cancer, active ingredients, key target genes, etc; "edges" represent the correlation between *C. reticulata* and *P. ternata* with their active components, target genes and non-small cell lung cancer, target genes and active components. The system network analysis can explore the pharmacological mechanism of *C. reticulata* and *P. ternata* in the treatment of non-small cell lung cancer.

Protein interaction (PPI) network

SSRING database is mainly used to predict the interaction between proteins and known proteins for searching. Through this database, 2031 species, including 13.8 million proteins and 9.6 million proteins, can be used to establish protein-protein interaction (PPI) network (34, 35). The target gene data of C. reticulata and P. ternata in the treatment of non-small cell lung cancer were imported into STRING database, and PPI network was constructed within the scope of "home sapiens". In the process of network construction, we can set a threshold for the confidence of protein-protein interaction. In this study, we selected moderate confidence of 0.4 to screen PPI.

Enrichment analysis of Gene Ontology (GO) and genome Encyclopedia (KEGG)

R language is a free and open-source software belonging to the GNU system. It is an excellent tool

commonly used in bioinformatics analysis and visualization. Bioconductor-related software package was installed in R software (version 3.6.2), and the data was transformed into gene name (Entrez ID) (36, 37). P < 0.05 and Q < 0.05 were defined to carry out enrichment analysis of GO and KEGG, and the results were output in the form of bubble chart and bar chart.

Statistical analysis

The measurement data were expressed in the form of mean ± standard deviation (SD). The experimental data was analyzed through SPSS20.0 software (SPSS, Inc., Chicago, IL, USA). The Legacy Dialogs analysis was used for non-normal data, Kruskal–Wallis H analysis was used to compare intergroup data, and Mann-Whitney U analysis was used to compare values between groups. P<0.05 was regarded as statistically significant in all experiments.

Results and discussion

Active components and target genes of C. reticulata and Pinellia Ternata

A total of 63 components of C. reticulata and 116 components of P. ternata were obtained by TCMSP database. According to DL ≥0.18 and OB≥30%, 5 active components of C. reticulata and 13 active components of P. ternata were obtained, and 18 active components of C. reticulata and P. ternata were obtained, as shown in Table 1. By selecting "Related Targeted", 479 target genes related to Citrus Reticulata, 1302 target genes related to P. ternata and 1781 target genes related to C. reticulata and P. ternata were retrieved. A total of 288 target genes correspond to the active components of "*C. reticulata* and *P. ternata*".

Related genes of non-small cell lung cancer (NSCLC)

According to "Non-small cell lung cancer (NSCLC)" as the keyword, 5236 Non-small cell lung cancer (NSCLC) related genes were retrieved from Genecards database, and then they were mapped with the target genes corresponding to the active ingredients of "C. reticulata and Pinellia Ternata", and 56 target genes of "C. reticulata and Pinellia Ternata" for the treatment of Non-small cell lung cancer (NSCLC) were obtained.

Table 1. Data list of active ingredients, OB and DL of *C. reticulata* and *P. Ternata.*

Name	Mol ID.	OB(%)	DL	Pharmaceutical ingredients
C. reticulata and P. ternata	MOL00359	36.91	0.75	sitosterol
	MOL00328	59.29	0.21	naringenin
	MOL005100	47.74	0.27	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl) chroman-4-one
	MOL005815	86.9	0.51	Citromitin
	MOL005828	61.67	0.52	noboletin
	MOL001755	36.08	0.76	24-Ethylcholest-4-en-3-one
	MOL002670	35.64	0.81	Cavidine
	MOL002714	33.52	0.21	baicalein
	MOL002776	40.12	0.75	Baicalin
	MOL000358	36.91	0.75	beta-sitosterol
	MOL000449	43.83	0.76	Stigmasterol
	MOL005030	30.7	0.2	gondoic acid
	MOL000519	31.11	0.32	coniferin
	MOL006936	39.99	0.2	10,13-eicosadienoic
	MOL006937	42.15	0.24	12,13-epoxy-9-hydroxynonadeca-7, 10-dienoic acid
	MOL006957	46.89	0.27	(3S,6S)-3-(benzyl)-6-(4-hydroxybenzyl) Piperazine-2,5-quinone
	MOL003578	38.69	0.78	Cycloartenol
	MOL006967	44.72	0.21	Beta-D-Ribofuranoside, xanthine-9

The "Drug-Component-Target-Disease" network

The data of active components, target genes of components and target genes related to Non-small cell lung cancer(NSCLC) patients of "C. reticulata and Pinellia Ternata" were imported into Cytoscape software to construct the "Drug-Component-Target-Disease" network.

Construction of PPI network

The target gene of "C. reticulata and Pinellia Ternata" for the treatment of Non-small cell lung cancer(NSCLC) was input into STRING, and the PPI network was constructed with medium confidence of 0.4. PPI network showed the interaction between proteins encoded by target genes. R language is used to extract the core genes with the closest interaction between proteins, which are represented by a horizontal bar graph. The top five genes were TP53, ESR1, FOS, NCOA3 and MAPK8 (Figure 1).

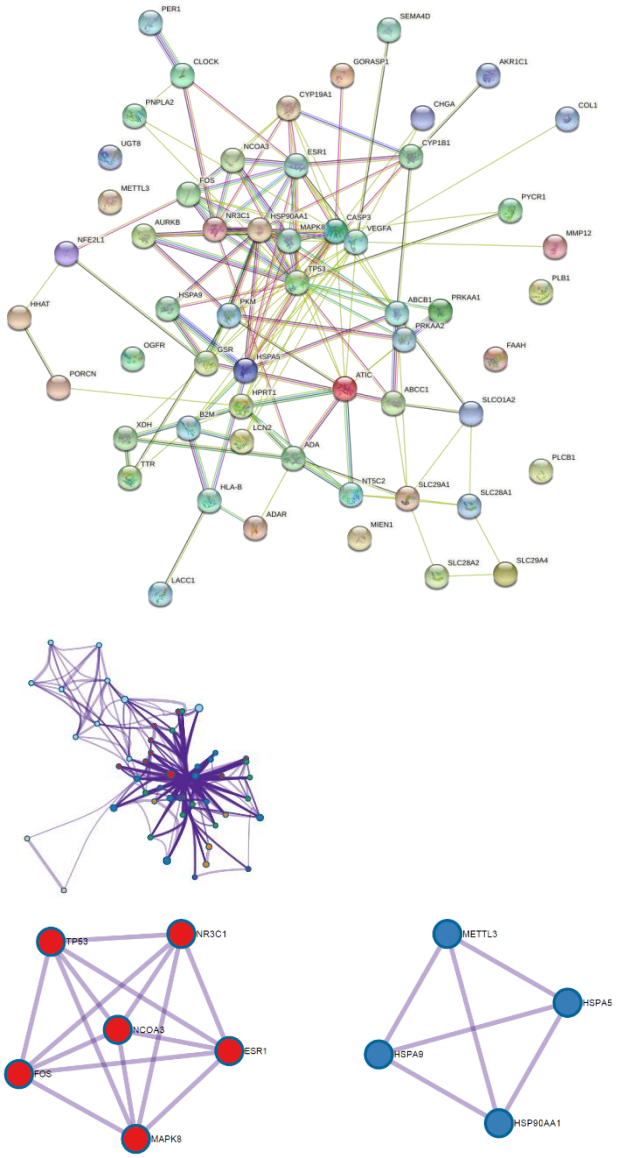
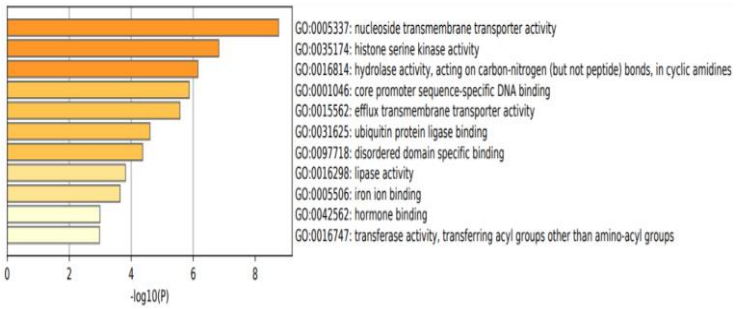


Figure 1. Construction of PPI network: The target gene of "*C. reticulata* and *P. ternata*" for the treatment of Non-small cell lung cancer (NSCLC) was input into STRING and the PPI network**.**

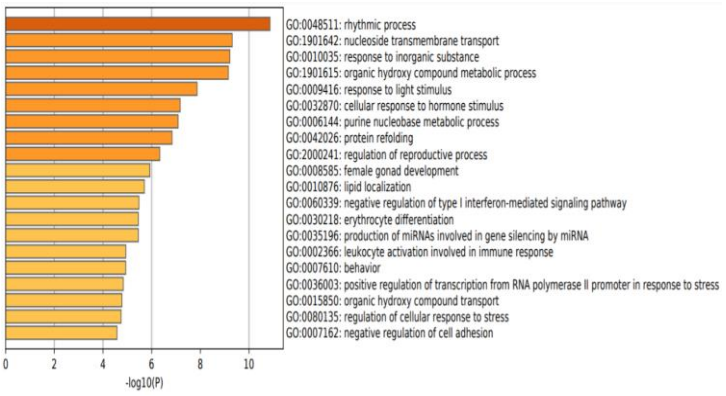
GO enrichment analysis

The functional enrichment analysis of "C. reticulata and Pinellia Ternata" on the core target genes was realized by R language, which involved 77 biological processes and was sorted by p-value correction, and the top 20 of them were represented by bar graph. The "C. reticulata and Pinellia Ternata" drug pair is mainly involved in transcription factor activity, nuclear receptor function, acetylcholine receptor activity, steroid hormone receptor activity, G protein-coupled amine receptor activity, neurotransmitter receptor activity, RNA polymerase II transcription factor binding and so on (Figure 2).

GO Molecular Functions



GO Biological Processes



GO Cellular Components

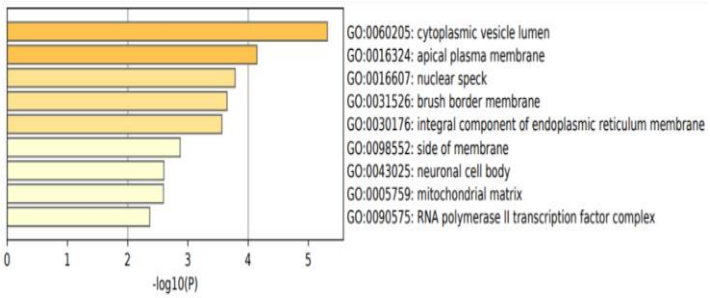


Figure 2. GO enrichment analysis: The functional enrichment analysis of "*C. reticulata* and *P. ternata*" on the core target genes was realized by R language.

KEGG enrichment analysis

The KEGG function enrichment analysis of the "C. reticulata and Pinellia Ternata" drug pair on core target genes was realized by R language. 82 signal pathways were involved in the treatment of non-small cell lung cancer (NSCLC). The corrected P values were sorted, and the top 20 were selected and represented by the horizontal bar chart. They are involved in the IL-17 signaling pathway, antigen processing and presentation, microRNAs in cancer and endocrine resistance and other signaling pathways, as well as colorectal cancer, prostate cancer

and other tumor-related signaling pathways (Figure 3).

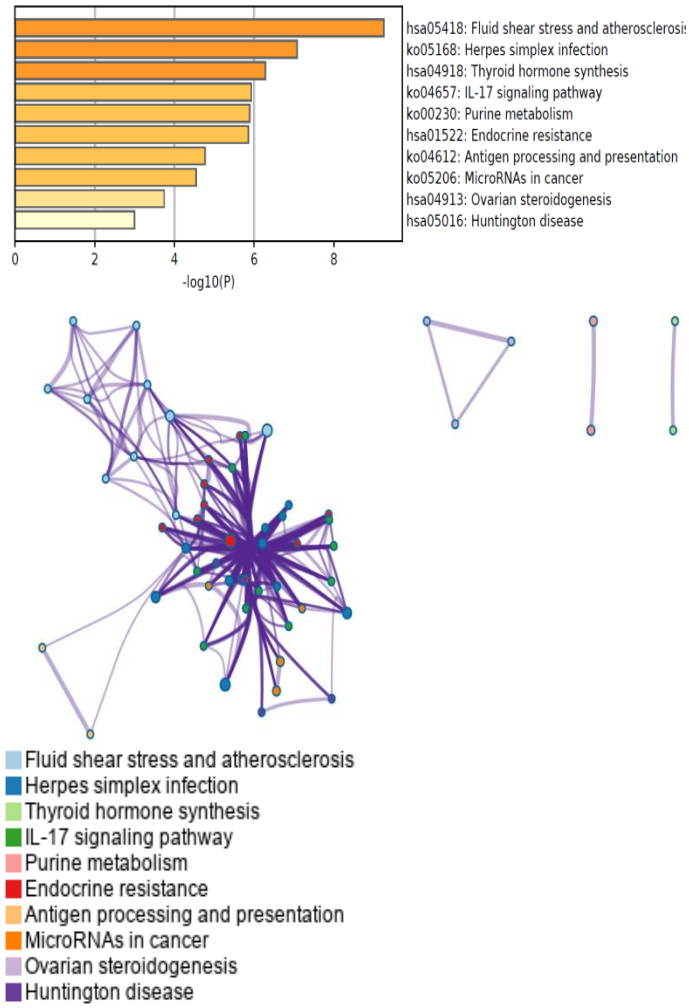


Figure 3. KEGG enrichment analysis: The KEGG function enrichment analysis of "*C. reticulata* and *P. ternata*" drug pair on core target genes was realized by R language.

The pathogenesis of non-small cell lung cancer tends to be lung dysfunction, phlegm coagulation and qi stagnation (19, 23). Qi Stagnation is a pattern of disharmony in Chinese Medicine, Qi can stagnate otherwise known as blocked due to stress of anxiety. Stagnation leads to slowing of blood flow, which means it can manifest as many different health issues.

Therefore, phlegm dampness obstructing the lung is one of the main pathological mechanisms. This paper discusses the process of this kind of pathogenesis. The famous medical books indicated that the airway of the human being is clear and smooth, and the phlegm is not produced (38). If it is choked, phlegm will be choked. Because of wind, cold, dampness and heat, and seven emotions, diet internal injury, resulting in Qi inverse fluid turbidity, and become the origin of various diseases. C. reticulata and P. ternata are commonly used to regulate qi and dissipate phlegm (13). Therefore, the mechanism of C. reticulata and P. ternata in the treatment of lung cancer with phlegm syndrome is worthy of further study. In this study, the main active components of C. reticulata and P. ternata were naringenin, baicalein, baicalin β- Sitosterol, coniferin, etc (16, 33). Studies have shown that naringenin can inhibit the proliferation and induce apoptosis of human lung cancer A549 cells by inducing the expression of death receptor (DR)5(39, 40). Naringenin can induce G1 phase arrest in NCIH 2170 cells, reduce the expression of poly ADP ribose polymerase (PARP), and inhibit the proliferation of human lung squamous cell carcinoma NCIH2170 cells (38, 41). At the same time, it can also up-regulate the expression of the apoptosis-inducing protein (BIB) in the BH3 domain, down-regulate the expression of the BCL-2 gene, activate procaspase-3 and procaspase-8, and induce the apoptosis of NCIH 2170 cells (42, 43). It was found that naringenin could significantly reduce the number of tumor cells metastasizing to the lung. It can prevent the occurrence and development of various tumors and inhibit the proliferation of various tumor cells. Baicalin can block the cell cycle and inhibit the proliferation of tumor cells to achieve a direct anti-tumor effect. At the same time, its antioxidant and anti-inflammatory effects can directly affect the proliferation of tumor cells. From the "Drug-Component-Target-Disease" network, β-Sitosterol acts on CASP9, CASP3, CASP8 and other targets closely related to apoptosis, so it may be effective in the treatment of Non-small cell lung cancer (NSCLC), which is worthy of further pharmacological study. Among them, TP53, ESR1, FOS, NCOA3 and MAPK8 are at the core, which are closely related to other proteins. VEGF is closely related to the proliferation, growth, migration, differentiation and apoptosis of tumor cells, and then affects the metastasis and prognosis of lung cancer (44, 45). The polymorphism of the ESR1 gene is associated with lung cancer in non-smoking women (46). The positive rate of c-FOS expression in non-small cell lung cancer was significantly higher than that in adjacent tissues, and its expression was related to lymph node metastasis. NCOA3 is closely related

to apoptosis and affects the occurrence, development and chemotherapy efficacy of tumors (47, 48). The enrichment of GO function was concentrated in transcription factor activity, nuclear receptor function, acetylcholine receptor activity, steroid hormone receptor activity, G protein-coupled amine receptor activity, neurotransmitter receptor activity, RNA polymerase II transcription factor binding and so on. KEGG function is enriched in the IL-17 signaling pathway, antigen processing and presentation, microRNAs in cancer and endocrine resistance and other signaling pathways. P53 is a tumor suppressor gene, which can promote the apoptosis of lung cancer cells by up-regulating the expression of p53 and its downstream genes. IL-17A/IL-17RA can up-regulate the expression of MMP-2 and MMP-9 by activating p38 MAPK signaling pathway in non-small cell lung cancer cells, which can affect the malignant biological behavior of non-small cell lung cancer and promote its progress. Therefore, the mechanism of C. reticulata and P. ternata in the treatment of non-small cell lung cancer may be closely related to the above signaling pathways. Network pharmacology is a kind of drug design method, which can obtain the correlation between active ingredients and target genes more efficiently. However, this method can not accurately obtain the regulatory relationship between active ingredients and target genes, which still needs to be verified by experiments.

Conclusion

Our study demonstrated that the main active components of C. reticulata and P. ternata in the treatment of non-small cell lung cancer were naringenin, baicalein, baicalin, baicalin β- Sitosterol, coniferin, etc. At present, there are few studies on β-sitosterol, which is worthy of further pharmacological study. The mechanism of C. reticulata and P. ternata on non-small cell lung cancer may be closely related to the expression of TP53, ESR1, FOS, NCOA3 and MAPK8, and may be related to the regulation of the IL-17 signaling pathway, antigen processing and presentation, microRNAs in cancer and endocrine resistance and other signaling pathways.

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