Network Pharmacology and Experiment Verification to Explore the Potential Mechanism of Yin-Huo-Tang for Lung Adenocarcinoma Recurrence

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Research

Keywords: Yin-Huo-Tang, Lung adenocarcinoma recurrence, network pharmacology, Sphingolipid signaling pathway, Traditional Chinese medicine

Posted Date: October 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-956330/v1>

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Abstract

Background

Lung adenocarcinoma (LUAD) is one of the most common malignancies with a rise in new cases worldwide each year. Recurrence significantly influences the survival in patients with LUAD. Yin-Huo-Tang (YHT) is a classic traditional Chinese prescription, used to prevent lung cancer relapse by “nourishing yin and clearing heat”.

Methods

In this study, the mechanism of YHT in LUAD recurrence was investigated. Firstly, the bioactive compounds-targets network and the protein–protein interaction network were constructed, and functional annotation and pathway enrichment analyses were performed. Pivotal compounds and hub genes were selected from the networks. Subsequently, the effectiveness of YHT was confirmed in lewis lung carcinoma mice. RNA sequencing was used to explore the mRNA expression differences between tumor tissues in the model mice and YHT-treated mice. The pathways screened by network pharmacology and RNA sequencing analysis at the same time were considered the most important pathways. At last, qualitative phytochemical analysis, molecular docking technology, PCR and WB analysis were used to validate the pivotal active ingredients, hub genes and main pathways.

Results

There were 128 active compounds, 419 targets interacting with LUAD recurrence. Network analysis identified 4 pivotal compounds, 28 hub genes and 30 main pathways. Target genes mainly focused on inflammation, metabolism, immune responses and apoptosis. We confirmed that YHT could inhibit the recurrence of lung adenocarcinoma through animal experimental study. Sphingolipid signaling pathway was the common main pathway in network pharmacology and RNA sequencing results. The hub genes related with the sphingolipid signaling pathway was S1PR5. Qualitative phytochemical analysis of the water extract of YHT confirmed the presence of 3 pivotal compounds, namely stigmasterol, nootkatone and ergotamine. The results of molecular docking verified the pivotal compounds of YHT could good affinity with the S1PR5. The PCR and WB analysis verified YHT suppressed lewis lung cancer cells proliferation by inhibiting S1P/S1PR5/Gi/Ras/Raf/MEK/ERK pathway, and inhibited migration through S1P/S1PR5/Gi/PI3K/RAC pathway.

Conclusion

The results confirmed the therapeutic effect of YHT on the recurrence of LUAD by multi-component-multi-target mode, the sphingolipid signaling pathway was one of the most relevant potential signaling pathways.

Background

Lung adenocarcinoma (LUAD) is one of the most common malignancies with a rise in new cases worldwide each year. Early diagnosis and surgical treatment are of great importance [1]. Undergoing surgery for early-stage patients and performing adjuvant treatments according to the conditions of the patient are recommended, but about 30-50% of early-stage lung cancer patients would die within 5 years of recurrent diseases [2]. The pathogenesis of LUAD recurrence is closely related to lipid metabolism alterations [3], immune infiltration [4, 5], gene mutations [6, 7], cell proliferation [5], enhanced stemness, DNA repair deficiency, bacterial microbiome [8] and angiogenesis [9, 10]. In the theory of traditional Chinese medicine (TCM), LUAD belongs to the category of “lung obstruction”, which can be treated by “nourishing yin and clearing heat”. Various Chinese herbal medicines and decoctions, such as *Rabdosia rosthornii* (Diels) H.Hara [11], *Tripterygium* Hook.f. [12], Ze-Qi-Tang [13] and Fei-Liu-Ping ointment [14] have been reported to inhibit autophagy and apoptosis of tumor cell, angiogenesis and epithelial to mesenchymal transition (EMT) outside the tumor [15, 16]. Thus, TCM has advantage in suppressing recurrence of lung cancer postoperative patients [17, 18].

Yin-Huo-Tang (YHT) is famous for clearing “heat” and nourish “yin” in TCM, which is first published in Chen shiduo’s “Bian Zheng Qi Wen” of the Chinese Qing Dynasty. It consists of the *Rehmannia glutinosa* (Gaertn.) DC. (Chinese name:Shudi), the *Morinda officinalis* F.C.How (Chinese name:Bajitian), the *Poria cocos* (Schw.) Wolf (Chinese name: Fuling), the *Ophiopogon japonicus* (Thunb.) Ker Gawl. (Chinese name:Maidong) and the *Schisandra chinensis* (Turcz.) Baill. (Chinese name:Wuweizi). Currently, YHT is used to treat lung cancer [19–21] based on the function of “nourishing yin and clearing heat”. However, the mechanism of YHT for treatment of LUAD remains unclear, impeding progress in clinical usage of YHT. Further studies are therefore necessary to explore the mechanisms involved.

Network pharmacology is an emerging strategy based on multi-disciplinary technologies. In this approach, the multiingredient-multitargets mechanism of herbs can be revealed systematically [22, 23].

In the study, the bioactive compounds-targets network and the protein–protein interaction (PPI) network were firstly constructed, then Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were performed[24], in order to find the pivotal compounds, hub genes, functional annotation and main pathways, which elucidated the molecular mechanism of YHT during LUAD recurrence. Subsequently, lewis lung carcinoma mice were used to confirm the effectiveness of YHT. And the differential expression genes (DEGs) induced by YHT therapy was analyzed by RNA sequencing in lewis lung cancer mouse model. KEGG enrichment also played on DEGs in order to find the common main pathways. At last, we proved the presence of pivotal compounds by qualitative phytochemical analysis, confirmed the hub genes which related with the main pathway by molecular docking, and verified the main pathway through PCR and WB analysis (Figure 1).

Methods

Methods

Candidate Compounds and Targets Screening

All of the chemical ingredients and targets of YHT were collected from the Traditional Chinese Medicine Systems Pharmacology database and Analysis Platform (TCMSP, <http://tcmssp.com>, updated in May 2020), Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (BATMAN-TCM, <http://bionet.ncpsb.org.cn/batman-tcm/>, updated in May 2020) and wide-scale searches of the literature [24]. Here, we selected as candidate compounds and targets that met the criteria of oral bioavailability (30 %) and drug similarity (0.14) in TCMSP, and score cutoff > 30, adjusted $p < 0.05$ in BATMAN-TCM for further analysis.

Identification of Disease Targets

We retrieved LUAD recurrent protein targets from GeneCards (<https://www.genecards.org/>, updated in May 2020) and OMIM database (<https://omim.org/>, updated in May 2020) [24]. Overlapping targets from drug and disease were obtained for further study. All the targets were standardized through UniProt [25].

Functional annotation and pathway enrichment analyses

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v.6.8 (<https://david.ncifcrf.gov>) was used for GO and KEGG enrichment analyses of the detected targets [24]. P value < 0.05 was set as statistically significant.

PPI Data

We used the Search Tool for the Retrieval of Interacting Genes (STRING, www.string-db.org/) database to construct a PPI network of the detected targets. And interaction score > 0.9 was considered statistically significant. Furthermore, the result of the PPI network was imported into the Cytoscape (version 3.7.1) plugin to create network visualizations and screened the hub genes with cytoHubba plugin [26, 27].

Network Construction

Cytoscape (version 3.7.1) software was used to construct drug-active ingredients- hub genes-disease network diagrams.

Preparation and Qualitative Phytochemical Analysis of YHT

YHT granule was purchased from Beijing Tcmages Pharmaceutical Co., LTD (Beijing, China). It consists of five common Chinese herbal extracts: *Rehmannia glutinosa* (Gaertn.) DC., the *Morinda officinalis* F.C.How, the *Poria cocos* (Schw.) Wolf, the *Ophiopogon japonicus* (Thunb.) Ker Gawl. and the *Schisandra chinensis* (Turcz.) Baill., as listed in Table 1. The five granules were mixed in water with a ratio of 30:10:10:5:2. Chromatographic separation was accomplished in an Thermo Ultimate 3000 system. We used the methods described in the Chinese Pharmacopoeia.

Table 1
Compositions of YHT

Latin name	Chinese name	Dose of herbal medicine(g)	Dose of granules(g)	Leaching rate of granules in water	Production and lot number
<i>Rehmannia glutinosa</i> (Gaertn.) DC.	Shudi	90	9	23.3%	Henan(China)19007601
<i>Morinda officinalis</i> F.C.How	Bajitian	30	3	10%	Guangdong(China)18031421
<i>Poria cocos</i> (Schw.) Wolf	Fuling	30	3	10%	Fuling(China)19014801
<i>Ophiopogon japonicus</i> (Thunb.) Ker Gawl.	Maidong	15	1.5	26%	Sichuan(China)19010501
<i>Schisandra chinensis</i> (Turcz.) Baill.	Wuweizi	6	0.6	NA*	Liaoning(China)19013262
* <i>Schisandra chinensis</i> (Turcz.) Baill. hardly soluble in water. This product is calculated as a dry product, and the content of schisandrol A is 0.1%.					

Chemical Reagents

Cisplatin was obtained from QILU PHARMACEUTICAL (Shandong, China).

Cell culture

Lewis lung cancer cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Animals and Experimental Groups

Fifty SPF C57BL/6 mice (50 males) weighing between 18 g and 22 g were purchased from the SPF (Beijing) Biotechnology Co.,Ltd. (Beijing China, License No. SYXK 2019-0010). The animal experiment was approved by the Animal Ethics Committee of the SPF (Beijing) Biotechnology Co.,Ltd. on November 15, 2020 (No. AW2020111501), as showed in Additional file 1: Figure S1.

The animals were randomly divided into five groups (n = 10/group): model (Mod), Cisplatin (Cis), YHT low-dose (YHT-L), YHT medium-dose (YHT-M) and YHT high-dose (YHT-H). The high dose is the dose documented in “Bian Zheng Qi Wen”. For the dose recorded in the book was already higher than the doses of other Chinese herbal medicine prescriptions, the ratio of the three doses of high, medium and low was 4:2:1.

Lewis lung cancer cells (2×10^6 in 200 μ L serum-free DMEM) were injected s.c. into the right armpit of mice. The subcutaneous tumors were removed 14 days after Lewis lung cancer cells injection. Then, all the mice were given the drug treatment for 14 days. The Mod group and Cis group received normal saline (10 ml/kg/d, intragastric). The Cis group was given cisplatin (3mg/kg/d, intraperitoneal injection) once a week, all the other groups were given the same volume of saline (intraperitoneal injection). Group YHT-L, YHT-M and YHT-H received YHT (5.5575g/kg/d, 11.115g/kg/d, and 22.23g/kg/d, respectively, intragastric, calculated by dose of herbal medicines). On day 28 after the injection of tumor cells, all animals were sacrificed to collect relevant samples.

Immunohistochemistry

Tumor tissues were completely stripped after all the animals had been sacrificed. One side of the tumor tissues was used for immunohistochemistry staining, according to methods described in previous study[28]. Primary antibody used was anti-Ki-67 (abcam, USA). The other tumor tissues were prepared for further analysis.

RNA Sequencing

RNA Sequencing was based on the Illumina Novaseq 6000 platform. The sequencing experiment used the Illumina Truseq™ RNA sample prep Kit method for library construction. DEGs between the tumor samples of Mod group and YHT-H group were screened by EdgeR. DAVID v.6.8 was used to perform KEGG pathway enrichment analysis.

Real-Time Quantitative Polymerase Chain Reaction (PCR)

Three tumor tissues were randomly selected from each group for real-time quantitative PCR, according to the methods described in our previous study [29]. The primer sequences of relative genes were listed in Table 2.

Table 2
The primer sequences of relative genes.

Gene	Forward	Reverse
β-actin	5'-CTACCTCATGAAGATCCTGACC-3'	5'-CACAGCTTCTCTTTGATGTCAC-3'
S1P	5'-GACTTCATGGATCATCCGTTTG-3'	5'-AAAAGCGAGCGATGTTATCTTC-3'
S1PR2	5'-TCTCTATGCTAAGCACTACGTG-3'	5'-GATGAAAACACCCAGTACGATG-3'
S1PR5	5'-TCTTGCTATTACTGGATGTCGC-3'	5'-GTGAAGGTGTAGATGATGGGAT-3'
Gi	5'-CTAAAGAGGCGAGGAAAGTCAG -3'	5'-GGATCCTCATCTGTTTGACGAT -3'
Ras	5'-TGCCTTCTAGAACAGTAGACAC-3'	5'-CTTTGCTGAGGTCTCAATGAAC-3'
Raf	5'-ACATCAACAACCGAGACCAGATCATC-3'	5'-CACAGTCAGCCACCAACCTCTTC-3'
MER	5'-GACTTTGAGAAGATCAGCGAAC-3'	5'-GTTTGATCTCCAGGTGGATCAG-3'
ERK	5'-ATCTCAACAAAGTTCGAGTTGC-3'	5'-ATGGTGTGCTCTGCTTATGATA-3'
PI3K	5'-AAACAAAGCGGAGAACCTATTG-3'	5'-TAATGACGCAATGCTTGACTTC-3'
RAC	5'-CCACTGTCCCAATACTCCTATC-3'	5'-CTTCTTCTCCTTCAGCTTCTCA-3'

Western Blot

Three tumor tissues were randomly selected from each group for western blot analysis, according to the methods described in our previous study [29]. Primary antibodies used were: S1P (Bioss, Beijing, China); S1PR2 (Proteintech, Wuhan, China); S1PR5 (Proteintech, Wuhan, China); GNAI1 (Proteintech, Wuhan, China); Rac1 (Proteintech, Wuhan, China); KRAS1 (Proteintech, Wuhan, China); ERK1 (Proteintech, Wuhan, China); RAF1 (Proteintech, Wuhan, China); Phospho-ERK1 (Proteintech, Wuhan, China); MEK-1 (Santa, USA); Phospho-Raf1 (abcam, USA); Phospho-MEK1 (abcam USA); actin (Proteintech, Wuhan, China).

Molecular Docking

The two-dimensional structures of the pivotal compounds were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). ChemBio3D (version 19.0.0.22) software was used to get three-dimensional structures of the pivotal compounds, and the structure was optimized according to minimize energy (minimum RMS gradient was 0.01). The crystal structure of the protein was obtained from RCSB PDB (<https://www.rcsb.org/>). The solvent and organic of the protein were removed by PyMOL. AutoDock Tools (version 1.5.6) was used to isolate protein and to get active grid box. The compound and protein were docked by Vina, and presented by PyMOL. The binding energy < -5.0 kal/mol was considered to have a stable binding conformation.

Statistical Analysis

The calculated results were mean ± standard deviation of at least 3 independent experiments. All the statistical analysis was done using SPSS20.0 (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered

statistically significant. The differences between the groups were examined using ANOVA, chi-square test and two-tailed unpaired Student's t-test.

Results

Compound Information

A total of 149 candidate compounds were selected through TCMSP and BATMAN-TCM databases, of which 23 only from Bajitian, 12 only from Fuling, 16 only from Maidong, 78 only from WuWeizi, 1 common from Bajitian and Shudi, 17 common from Fuling and Wuweizi, 2 common from Shudi and Maidong, as listed in Additional file 2: Table S1.

Targets in YHT Active Against LUAD recurrence

A total of 111,170 targets directly and indirectly related to LUAD relapse were obtained and 487 YHT related objectives were obtained (Additional file 3: Table S2). A total of 419 shared targeted were obtained (Figure 2A, Additional file 3: Table S2).

There were 128 active ingredients correlated with the 419 common targets. Active ingredients-gene symbols network was shown in Additional file 4: Figure S2 (Additional file 5: Table S3). And four pivotal active ingredients were identified with degree ≥ 46 , namely stigmasterol, epiguaipyridine, nootkatone, ergotamine.

Construction of PPI network and identification of hub genes

The PPI relationship of 419 target genes was obtained with STRING tool, and the minimum interactive score was 0.9 (Figure 2B). The genes evaluated by maximal clique centrality (> 87178291119 , Figure 2C) and the degree (> 22 , Figure 2D) were identified: S1PR5, PTGER3, OPRM1, OPRK1, MTNR1B, MTNR1A, HTR1D, HTR1B, HTR1A, DRD4, DRD3, DRD2, CXCR4, CX3CR1, CNR2, CNR1, CHRM2, ANXA1, ADRA2C, ADRA2B, ADRA2A, ADORA3, ADORA1, ADCY1, ADRB2, F2, PIK3CA, PIK3R1.

Drug-Active ingredients-Hub Genes-Disease Network Analysis

Drug-active ingredients- hub genes-disease network was shown in Figure 3, and the more details were listed in Additional file 6: Table S4. There were 5 Chinese herbs, 48 active ingredients and 28 hub genes in the network.

GO and KEGG Pathway Enrichment Analyses

The 419 common target genes were enriched with GO and KEGG analysis. It turned out that the main biological processes were "oxidation-reduction process", "response to hypoxia", "regulation of postsynaptic membrane potential", "regulation of ion transmembrane transport", "G-protein coupled receptor signaling pathway" and "coupled to cyclic nucleotide second messenger"; the cellular

component terms were enriched in “plasma membrane”, “integral component of plasma membrane”, “voltage-gated sodium channel complex”, “GABA-A receptor complex and postsynaptic membrane”; the molecular function terms were enriched in “drug binding”, “extracellular ligand-gated ion channel activity”, “L-ascorbic acid binding”, “GABA-A receptor activity” and “voltage-gated sodium channel activity”. In terms of signaling pathway enrichment analysis, we found target genes mainly focused on inflammation, metabolism, immune responses, and apoptosis, such as “Purine metabolism (hsa00230)”, “AMPK signaling pathway (hsa04152)”, “Human cytomegalovirus infection (hsa05163)”, “Apoptosis (hsa04210)”, “Insulin signaling pathway (hsa04910)”, “Sphingolipid signaling pathway (hsa04071)”, “Human immunodeficiency virus 1 infection (hsa05170)” and “Human T-cell leukemia virus 1 infection (hsa05166)” (Figure 4).

Identification of the Chemical Constituents in Yin-Huo-Tang

Figure 5 given a typical total ion chromatograms (TICs) of non-volatile components extracted by YHT. Although TICs are complex, most chromatographic peaks can be well separated. Chemical compositions in YHT water extracts determined were shown in Additional file 7-9: Table S5-7. Three of the four pivotal active ingredients obtained from the network pharmacology analysis were identified through qualitative phytochemical analysis, namely stigmasterol, nootkatone and ergotamine.

In vivo Validation of the Efficacy of Yin-Huo-Tang

We established a lewis lung cancer mouse model and evaluated the potential therapeutic effect of YHT.

During the first 7 days of treatment, the weights of all animals gradually increased. During the last 7 days of treatment, the weights of the model and Cisplatin-treated mice decreased. During the last 3 days of treatment, the body weights of YHT-L, YHT-M and YHT-H mice decreased. On the tenth day, body weight of the Cis group was the lowest ($P < 0.05$, Figure 6). However, after 14 days of treatment, there was no significant difference, which was related to the increasing tumor burden. During the drug treatment, the tumor volumes of all mice gradually increased. On the fourteenth day, tumor volumes of Cisplatin-treated and YHT-H-treated mice were significantly lower than those of the model, YHT-M-treated and YHT-L-treated mice ($P < 0.05$, Figure 6). The number of tumor recurred mice were potentially reduced in the cisplatin and YHT-H treatment groups compared to the model group after treatment for 4 days (Table 3). After treatment for 7 days, the tumor had recurred in all mice, which was related to the high invasiveness of lewis lung carcinoma cells. As expected, cisplatin and YHT-H decreased Ki-67 (a marker of cell proliferation) in tumor tissues (Figure 7). We believed that high doses of YHT could significantly inhibit the recurrence of lung adenocarcinoma.

Table 3
Tumor recurrence information

Group	Recurrence rate on day 4	P value *	Recurrence rate on day 7	P value*
Mod	100%	-	100%	-
Cis	71.4%	0.008	100%	-
YHT-L	100%	-	100%	-
YHT-M	100%	-	100%	-
YHT-H	75%	0.003	100%	-
** versus Mod group				
<p>RNA sequencing was used to explore the mRNA expression differences between tumor tissues in the model mice and YHT-H-treated mice. There were 2099 differentially expressed genes, including 1041 upregulated and 1058 downregulated (Figure 8). KEGG analysis was used to analyze the functional enrichment of important modules. The top 15 potential signaling pathways were listed in Figure 8. "Sphingolipid signaling pathway" and "Non-alcoholic fatty liver disease (NAFLD) pathway" were predicted by RNA sequencing and network pharmacological analysis at the same time. Sphingolipid signaling pathway was related to cell survival proliferation, migration and cytoskeletal events (Figure 9). Then we verified the inhibition of YHT-H for Sphingolipid signaling pathway by PCR and WB analysis. As expected, YHT-H suppressed Lewis lung cancer cells proliferation by inhibiting S1P/S1PR5/Gi/Ras/Raf/MEK/ERK pathway, and inhibited migration through S1P/S1PR5/Gi/PI3K/RAC pathway (Figure 10). YHT could inhibit the recurrence of LUAD by modulating the Sphingolipid signaling pathway.</p>				

Molecular Docking

Sphingolipid signaling pathway was the common main pathways in network pharmacology and RNA sequencing results. S1PR5 was a key gene in Sphingolipid signaling pathway, and was a hub gene selected by network pharmacology analysis. We found 3-Phenyldecane (Compound CID: 20740), alpha-Cuparenol (Compound CID: 5316200), cuparene (Compound CID: 86895), 4-Phenylbicyclo [2.2.2] octan-1-ol (Compound CID: 327096) were related with S1PR5 according to network pharmacology analysis (Supplementary Table 4). Stigmasterol (MOL ID: MOL000449), epigallocatechin gallate (Compound CID: 5317082), nootkatone (Compound CID: 1268142), ergotamine (Compound CID: 8223) were the four pivotal active ingredients. The above 8 substances were docking with S1PR5 respectively. All of them could bind well to S1PR5, as showed in Figure 11, and the detail was listed in Table 4.

Table 4
Docking results.

Gene name	Active ingredients	Affinity (kcal/mol)
S1PR5(7EW1)	Stigmasterol	-7.2
	Epiguaipyridine	-6.5
	Nootkatone	-6.3
	Ergotamine	-10.2
	3-Phenyldecane	-4.8
	Alpha-Cuparenol	-6.8
	Cuparene	-6.6
	4-Phenylbicyclo	-6.6

Discussion

Tumor recurrence or metastasis is responsible for lung cancer death. It has been reported that TCM is potentially effective in the prevention of cancer relapse [30–32]. YHT is a classic traditional Chinese prescription used for prevention LUAD recurrence by “nourishing yin and clearing heat”. In this study, we predicted the possible pharmacological mechanism of YHT through a network pharmacology approach, and carried out experiment applying lewis lung carcinoma mice to identify it.

Through a web-based pharmacology approach, 128 potential active ingredients and 419 genetic targets related to LUAD recurrence were identified. S1PR5, PTGER3, OPRM1, OPRK1, MTNR1B, MTNR1A, HTR1D, HTR1B, HTR1A, DRD4, DRD3, DRD2, CXCR4, CX3CR1, CNR2, CNR1, CHRM2, ANXA1, ADRA2C, ADRA2B, ADRA2A, ADORA3, ADORA1, ADCY1, ADRB2, F2, PIK3CA, PIK3R1 were considered to be hub genes (Figure 2). Stigmasterol, epiguaipyridine, nootkatone and ergotamine were identified as pivotal active ingredients. We also revealed several meaningful signaling pathways including “Purine metabolism”, “AMPK signaling pathway”, “Human cytomegalovirus infection”, “Apoptosis”, “Insulin signaling pathway”, “Sphingolipid signaling pathway” and “Human T-cell leukemia virus 1 infection” (Figure 4). These pathways are mainly related to inflammation, metabolism, immune responses, and apoptosis.

Qualitative phytochemical analysis of the water extract of YHT confirmed the presence of anthraquinone, polyol, phytosterol, terpene, aldehyde, carboxylic compounds, sugar acid, and vitamins (Figure 5). Three of the four pivotal active ingredients also existed in the water extract of YHT, namely stigmasterol, nootkatone and ergotamine. It has been reported that stigmasterol could cause several cancer cell apoptosis, such as ovarian cancer [33], gastric cancer [34] and cholangiocarcinoma [35]. However, the role of stigmasterol in lung cancer was rarely reported. Nootkatone inhibited growth of A549 cells and induced G1 cell cessation by activating AMPK via LKB1-independent and CAMKK2-dependent pathways [36]. Ergotamine was a vasoconstricting agent, which could cause toxicity and inhibit tumor growth [37].

The anti-tumor mechanism of ergotamine was unclear yet, and it was worthy of further investigation. There were some other active ingredients identified in the water extract of YHT had direct anticancer effects or inhibited recrudescence and metastasis. P-cymene inhibited A549 cell proliferation through DNA damaging [38]. Beta sitosterol perturbed disturbed cell cycle and induced apoptosis of A549 cells [39], and inhibited H1299 proliferation [40]. Ophiopogonin B inhibited metastasis of A549 cells via the linc00668/miR-432-5p/EMT axis [41]. Ophiopogonin D blocked proliferation of human lung-cancer cells through the suppression of NF- κ B, PI3K/AKT, and AP-1 pathways [42]. Pachymic acid induced apoptosis via activating ROS-dependent JNK and ER stress pathways in lung cancer cells [43]. Linalool inhibited A549 cell migration and induced cell growth inhibition and mitochondrial depolarization [44].

In vivo experiments confirmed YHT-H reduced the postoperative recurrence of lewis lung carcinoma and inhibited tumor cell proliferation (Figure 6,7). This dose was the dose documented in “Bian Zheng Qi Wen”. RNA sequencing was performed to elaborate the mechanism of YHT acting on lewis lung adenocarcinoma recurrence. RNA sequencing, mRNA and protein expression of tumor tissues confirmed sphingolipid signaling pathway was one of the most important mechanisms of YHT treating LUAD recurrence (Figure 8,9). S1P played an important role in normal physiology, inflammation and carcinogenesis [45]. S1P could act on S1P receptors to promote tumorigenesis, and operate between cancer cells and fibroblasts to prompt metastasis of cancer cells [46]. S1P receptors unusual expressed in various cancers, such as colon cancer [47], bladder cancer [48], oral cancer [49]. And it had been reported that S1PR5 had the highest expression level among all the S1P receptors in malignant human tissues [50]. The mitogen-activated protein kinase (MAPK) pathway was a key cell signaling pathway involved in regulating cellular growth, proliferation, and survival. The RAC1 was critical for cellular adhesion, migration, motility and cell proliferation [51]. PI3K/AKT signaling pathway induced RAC1 expression and activity, and then enhanced cell proliferation, survival, migration and metastasis [52]. MAPK pathway [53–55] and RAC1 [56, 57] were the focuses of the development of new anti-tumor drugs. YHT-H suppressed lewis lung cancer cells proliferation by inhibiting S1P/S1PR5/Gi/Ras/Raf/MEK/ERK pathway, and inhibited migration through S1P/S1PR5/Gi/PI3K/RAC pathway (Figure 10).

Molecular Docking showed S1PR5 could docking well with the four pivotal active ingredients and other four active ingredients obtained from TCMSP and BATMAN-TCM databases. These further proved that YHT suppressed lung cancer recurrence by inhibiting the sphingolipid signaling pathway. Combined with the results of qualitative phytochemical analysis, we believed stigmasterol, nootkatone and ergotamine played significant roles.

Conclusions

In conclusion, the present study demonstrated the anticancer effect of YHT. To explore the biological mechanism of YHT inhibiting the recurrence of lung adenocarcinoma we did network pharmacology analysis. We concluded that Stigmasterol, nootkatone and ergotamine were the most important pivotal active ingredients. We found 28 hub genes including S1PR5. The mechanism of YHT could be summed up to inflammation, metabolism, immune responses, and apoptosis. Notably, in the enrichment analysis

of main target genes according to the network pharmacological analysis and differentially regulated makers acquired from Lewis lung carcinoma mice, sphingolipid signaling pathway was found to be one of the shared pathways. Then we verified YHT suppressing tumor cells proliferation and inhibiting migration through sphingolipid signaling pathway. And Stigmasterol, nootkatone and ergotamine could dock well with S1PR5. These findings provide a new option for the treatment of LUAD recurrence and also provide information for further revealing the mechanisms of YHT (Figure 1).

However, our study was based on network pharmacology research and limited animal experimental research. Cell model and clinical samples were not used to further verify its authenticity. The pivotal active ingredients were verified by qualitative phytochemical analysis and molecular docking, lacked of quantitative analysis and experimental verification. Our discovery should be further validated in future study.

Abbreviations

LUAD: Lung Adenocarcinoma; YHT: Yin-Huo-Tang; TCM: Traditional Chinese Medicine; EMT: Epithelial to Mesenchymal Transition; PPI: Protein–Protein Interaction; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: The Differential Expression Genes; TCMSP: The Traditional Chinese Medicine Systems Pharmacology database and Analysis Platform; BATMAN-TCM: Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine; DAVID: The Database for Annotation, Visualization, and Integrated Discovery; STRING: The Search Tool for the Retrieval of Interacting Genes; TICs: Typical total ion chromatograms.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets of RNA sequencing analyzed during the current study are available in the sequence read archive (SRA) repository, named PRJNA761925 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA761925>).

The other original contributions presented in the study are included in the article/Supplementary Information, further inquiries can be directed to the corresponding authors.

Competing interests

The authors declare that they have no conflicts of interest.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities (No. 2020-JYB-ZDGG-127), the National Key R&D Program of China (No. 2018YFC1705102) and Capital's Funds for Health Improvement and Research (No. CFH 2018-1-4201).

Authors' contributions

Dianna Liu, Kaiwen Hu and Quanwang Li designed the experiments. Dianna Liu, Shicheng Lin and Yuan Li participated in the specific experimental process. Dianna Liu and Tian Zhou performed the research and analyzed the data. Dianna Liu wrote the paper. Dianna Liu, Kaiwen Hu, and Quanwang Li drafted the manuscript. All authors approved and agreed to be responsible for all aspects of the work.

Acknowledgements

Not applicable.

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Figures

Figure 1

The technical strategy. Firstly, YHT bioactive compounds and related targets were preliminarily understood, and drug-active ingredients-hub gene symbols-disease network was constructed, in order to elucidate their molecular mechanism during LUAD recurrence. Subsequently, the efficacy of YHT was validated in lewis lung carcinoma mice. Meanwhile, the RNA sequencing was used to explore DEGs thus providing the potential mechanisms. Sphingolipid signaling pathway was predicted by RNA

sequencing and network pharmacological analysis at the same time. Finally, qualitative phytochemical analysis, molecular docking technology, PCR and WB analysis were used to validate the pivotal active ingredients (Stigmasterol, nootkatone and ergotamine), hub gene (S1PR5) and main pathway (Sphingolipid signaling pathway).

Figure 2

PPI networks. (A) 419 relevant overlapping targets shared between YHT and LUAD recurrence;(B) PPI network of YHT acting on LUAD recurrence (the minimum interaction score was 0.9); (C) and (D) The hub genes evaluated by maximal clique centrality (> 87178291119, Figure 2C) and the degree (> 22, Figure 2D).

Figure 3

Drug-Active ingredients-Hub gene symbols-Disease network of YHT in the treatment of LUAD recurrence. The detail of the code in the figure was listed in Additional file 6: Table S4. There were 5 Chinese herbs, 48 active ingredients and 28 hub genes in the network.

Figure 4

GO and KEGG enrichment analysis. (A) Barplot showing the BP, CC, and MF domains in the ontology; (B) Dotplot showing the top 30 of KEGG pathways.

Figure 5

TICs of the water extract of YHT by UPLC/Q-TOF-MS/MS. (A) TIC of YHT in positive ion mode; (B) TIC of YHT in negative ion mode.

Figure 6

Body weight, tumor volume of mice. (A) and (B) On the tenth day, body weight of the Cis group was the lowest. However, there was no significant difference among them after drug treatment for 14 days; (C) and (D) After drug treatment for 14 days, tumor volumes of Cisplatin-treated and YHT-H-treated mouse were significantly lower than those of the model, YHT-M-treated and YHT-L-treated mice. Error bars are means $\pm$ SD, $n = 10$. P values by one-way analysis of variance. * $P < 0.05$ and ** $P < 0.01$.

Figure 7

Immunohistochemical staining (Ki67) of tumor tissues from Lewis lung carcinoma mice with the indicated treatments. Error bars are means $\pm$ SD, n= 3 randomly selected magnification fields. P values by one-way analysis of variance. *P<0.05 and **P<0.01 .

Figure 8

The results of RNA sequencing between tumor tissues in the model mice and YHT-H-treated mice. (A)Volcano plot of the DEGs between tumor tissues in the model mice and YHT-H-treated mice; (B) Dotplot showing the top 15 of KEGG pathways. “Sphingolipid signaling pathway” and “Non-alcoholic fatty liver disease (NAFLD) pathway” were predicted by RNA sequencing and network pharmacological analysis at the same time.

Figure 10

YHT suppressed lewis lung carcinoma recurrence by inhibiting the sphingolipid signaling pathway. (A) and (B) qRT-PCR analysis of S1P, S1PR5, Gi, PI3K, RAC, Ras, Raf, MEK and ERK expression in lewis lung carcinoma mice after the indicated 14 days treatments; (C) Western blotting analysis of S1P, S1PR5, Gi, PI3K, RAC, Ras, Raf, MEK and ERK expression in lewis lung carcinoma mice after the indicated 14 days treatments. Error bars are means $\pm$ SD, $n = 3$ randomly selected magnification fields. P values were calculated using two-tailed unpaired Student's t-test. * $P < 0.05$ and ** $P < 0.01$.

Figure 11

Molecular Docking. S1PR5 could docking well with the four pivotal active ingredients and other four active ingredients obtained from TCMSP and BATMAN-TCM databases.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1EthicsStatementofAnimalExperimentStudy.pdf](#)
- [FigureS2ActiveingredientsTargetNetwork.tif](#)
- [TableS1CandidateCcompoundsofYHT.xlsx](#)
- [TableS2TargetsofYHTandLUADRecurrence.xlsx](#)
- [TableS3DrugActiveingredientsGenesDiseaseNetwork.csv](#)
- [TableS4DrugActiveingredientsHubgenesDiseasenetwork.csv](#)
- [TableS5LCMSAnalysisofAll.xlsx](#)
- [TableS6LCMSAnalysisofMetabolomepos.xlsx](#)
- [TableS7LCMSAnalysisofMetabolomeneg.xlsx](#)