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Research paper

Integrating network pharmacology and bioinformatics analysis to explore the mechanism of Yupingfengsan in treating lung adenocarcinoma

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ABSTRACT

Introduction: Yupingfengsan (玉屏风散) is a classical traditional Chinese formulae with proven therapeutic effect on the disease of respiratory system. However, its action mechanism remains obscure for lung adenocarcinoma (LAD). This study aimed to investigate the potential target genes and the mechanism of Yupingfengsan in the treatment of LAD.

Methods: Yupingfengsan chemical ingredients and related targets were predicted by public databases. Differential expression genes of LAD were obtained from The Cancer Genome Atlas (TCGA) database. Gene set enrichment analysis (GSEA) was used to execute gene ontology function and pathway enrichment analysis. The protein interaction network was constructed by Cytoscape. Finally, potential target genes and mechanism of Yupingfengsan on LAD were predicted. The differential expression and prognostic analysis of target genes were verified by TCGA database and the Kaplan-Meier plotter, respectively.

Results: A total of 253 targets from 30 active ingredients of Yupingfengsan were predicted. 4426 DEGs were identified from TCGA LAD data sets. 32 upregulated and 52 downregulated genes were obtained between Yupingfengsan putative targets and DEGs of LAD. MAPK, VEGF, and ErbB signaling pathways seemed to be the most likely mechanism. 6 potential target genes, PLA2G4A, PRKCB, PIK3R1, KDR, PLA2G1B and PTGS2 were extracted. These 6 candidates were verified significantly different expression between LAD and paired adjacent normal tissues, and PRKCB, PIK3R1, KDR, PLA2G1B suggested a significant correlation with LAD prognosis.

Conclusion: Differentially expressed in tumors, PLA2G4A, PRKCB, PIK3R1, KDR, PLA2G1B and PTGS2 are potential therapeutic targets of Yupingfengsan in the treatment of LAD, and MAPK, VEGF and ErbB signaling pathways are involved.

1. Introduction

Lung cancer remains the leading cause of cancer incidence and mortality in the worldwide, representing close to 1 in 5 (18.4%) cancer deaths [1,2], similar trends were observed in China [3]. Lung adenocarcinoma (LAD), which accounts for more than 50% lung cancer, is one of the most aggressive and rapidly fatal tumor types [4]. Targeted therapies have revealed the development of effective personalized treatment strategies [5–7]. In spite of this, nearly all patients eventually have disease progression due to acquired resistance. Immunotherapy has shown rapid and durable responses in around 20% of previously treated patients with advanced NSCLC [8–11]. However, the toxic profile of checkpoint inhibitors is quite favorable, with about 10% of patients developing severe (grades III-IV) [12,13]

Traditional Chinese medicine (TCM) has been widely used in China for thousands of years. In several clinical studies, there has been shown that Chinese herbs are an effective and safe treatment for lung [14], breast [15], gastric [16], liver [17], pancreatic [18], colorectal cancer [19]. The famous Chinese herbal formulae “Yupingfengsan” is commonly used for diseases of respiratory system, such as chronic obstructive pulmonary disease (COPD) [20], respiratory tract infections [21], and pneumonia [22]. In the aspect of treatment in lung carcinoma, Yupingfengsan has played an important role in adjuvant chemoradiotherapy in the treatment of lung carcinoma [23]. Yupingfengsan combined with chemoradiotherapy can significantly reduce the toxicity of anticancer therapy and improve quality of life. Unfortunately, little genomic research on Yupingfengsan is currently available, and the molecular mechanism of Yupingfengsan for lung

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Table 1
ADME parameters information of 30 ingredients from Yupingfengsan. Oral bioavailability (OB). Drug likeness (DL). Drug half-life (HL).Huangqi (HQ), Baizhu (BZ) and Fangfeng (FF).

Number	MOL_ID	Molecule_name	Source	OB	DL	HL
1	MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	HQ,BZ	36.23	0.78	5.22
2	MOL000098	quercetin	HQ	46.43	0.28	14.40
3	MOL000211	Mairin	HQ	55.38	0.78	8.87
4	MOL000239	Jaranol	HQ	50.83	0.29	15.50
5	MOL000296	hederagenin	HQ	36.91	0.75	5.35
6	MOL000354	isorhamnetin	HQ	49.60	0.31	14.34
7	MOL000371	3,9-di-O-methylnissolin	HQ	53.74	0.48	9.00
8	MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	HQ	36.74	0.92	13.06
9	MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	HQ	64.26	0.42	8.49
10	MOL000387	Bifendate	HQ	31.10	0.67	17.96
11	MOL000392	formononetin	HQ	69.67	0.21	17.04
12	MOL000398	isoflavanone	HQ	109.99	0.30	15.51
13	MOL000417	Calycosin	HQ	47.75	0.24	17.10
14	MOL000422	kaempferol	HQ	41.88	0.24	14.74
15	MOL000433	FA	HQ	68.96	0.71	24.81
16	MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	HQ	39.05	0.48	7.95
17	MOL000020	12-senecioyl-2E,8E,10E-atractylentriol	BZ	62.40	0.22	6.07
18	MOL000021	14-acetyl-12-senecioyl-2E,8E,10E-atractylentriol	BZ	60.31	0.31	5.32
19	MOL000022	14-acetyl-12-senecioyl-2E,8Z,10E-atractylentriol	BZ	63.37	0.30	6.43
20	MOL000072	8β-ethoxy atractylenolide III	BZ	35.95	0.21	8.34
21	MOL000359	sitosterol	FF	36.91	0.75	5.37
22	MOL011730	11-hydroxy-sec-o-beta-d-glucosylhamaudol_qt	FF	50.24	0.27	12.71
23	MOL011737	divaricatacid	FF	87.00	0.32	14.83
24	MOL011740	divaricatol	FF	31.65	0.38	13.41
25	MOL011747	ledebouriellol	FF	32.05	0.51	14.57
26	MOL011753	5-O-Methylvisamminol	FF	37.99	0.25	14.67
27	MOL000173	wogonin	FF	30.68	0.23	17.75
28	MOL000358	beta-sitosterol	FF	36.91	0.75	5.36
29	MOL001494	Mandenol	FF	42.00	0.19	5.39
30	MOL007514	methyl icosa-11,14-dienoate	FF	39.67	0.23	5.24

cancer has not been elucidated.

TCM exhibits therapeutic efficacy by the synergistic effects of "multi-component, multi-target, and multi-pathway" [24]. Additionally, Chinese herbal formulae always contain different kinds of herbs and have effects on many targets, therefore the anti-tumor mechanisms of herbal formulae are hard to understand. The network pharmacology, first proposed by Andrew L Hopkins [25,26], incorporating systems biology, bioinformatics, and polypharmacology. It is an effective approach to investigate the activity of ingredients and the targets of herbs in traditional Chinese herbal formulae [27]. Bioinformatics analysis is a powerful method that can aid in identifying important mechanisms for tumorigenesis, progression and treatment. High-throughput sequence provides strong tools to comprehensively quantify gene expression [28]. Integrated network pharmacology and bioinformatics analysis can assist us to explore mechanisms of traditional Chinese formulae for cancer.

In this study, we used a network pharmacology approach to predict the targets and mechanisms of Yupingfengsan in the treatment of LAD. Bioinformatics analysis was performed to verify the expression of potential target genes between LAD and adjacent normal tissue, the relationship of potential target genes and prognosis in LAD also conducted.

2. Materials and methods

2.1. Yupingfengsan ingredient collection and target identification

Yupingfengsan contains three Chinese herbs, Huangqi (HQ, Hedysarum Multijugum Maxim), Baizhu (BZ, Atractylodes Macrocephala Koidz), and Fangfeng (FF, Saposhnikoviae Radix). The chemical ingredients were collected from Traditional Chinese Medicine Systems Pharmacology (TCMSP, version 2.3, http://lsp.nwu.edu.cn/tcmsp.php) database [29]. High OB (oral bioavailability) is often a key indicator to determine the drug-like property of bioactive molecules as therapeutic agents [30]. The DL (drug likeness) level of the compounds is 0.18, which are used as a selection criteria for the 'drug-like' compounds in traditional Chinese herbs [31]. HL (Drug half-life) dictates for the timescale over which the compound may elicit therapeutic [32]. In this study, the ingredients were screened according to OB ≥ 30%, DL ≥ 0.18 and DL ≥ 4 h, which are suggested criteria of TCMSP database.

The compounds were unified into SMILES using Pubchem (https://pubchem.ncbi.nlm.nih.gov/) database, mol2 format was converted to SMILES by ALOGPS 2.1 online tool [33] (http://www.vcclab.org/) if the SMILES was not searched in the Pubchem database. Predicted targets were selected by SwissTargetPrediction [34,35] (http://www.swisstargetprediction.ch/) and SuperPred [36] (http://prediction.charite.de/), two online tools for predicting targets of a small molecule by using 2D or 3D similarity measures. The targets were merged and the repeated genes were removed. For further study, gene information was normalized into Uniprot ID (https://www.uniprot.org/).

2.2. Differential expression genes (DEGs) of LAD

mRNA profile of LAD was obtained from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database, significant genes were identified by GeneSpring GX 12.5 software (Agilent Technologies, Santa Clara, CA, US). Robust multi-array average (RMA) quantile normalization analysis algorithm was used to normalize gene expression data [37]. 3D principal component analysis (PCA) scores were performed for quality control [38]. T *t*-test unpaired analysis, Asymptotic p-value computation with Benjamini-Hochberg multiple testing correction, was utilized to detect DEGs whose fold change (FC) ≥ 2 with false discovery rate (FDR) cut-off < 0.05 between LAD and adjacent non-tumor tissues.

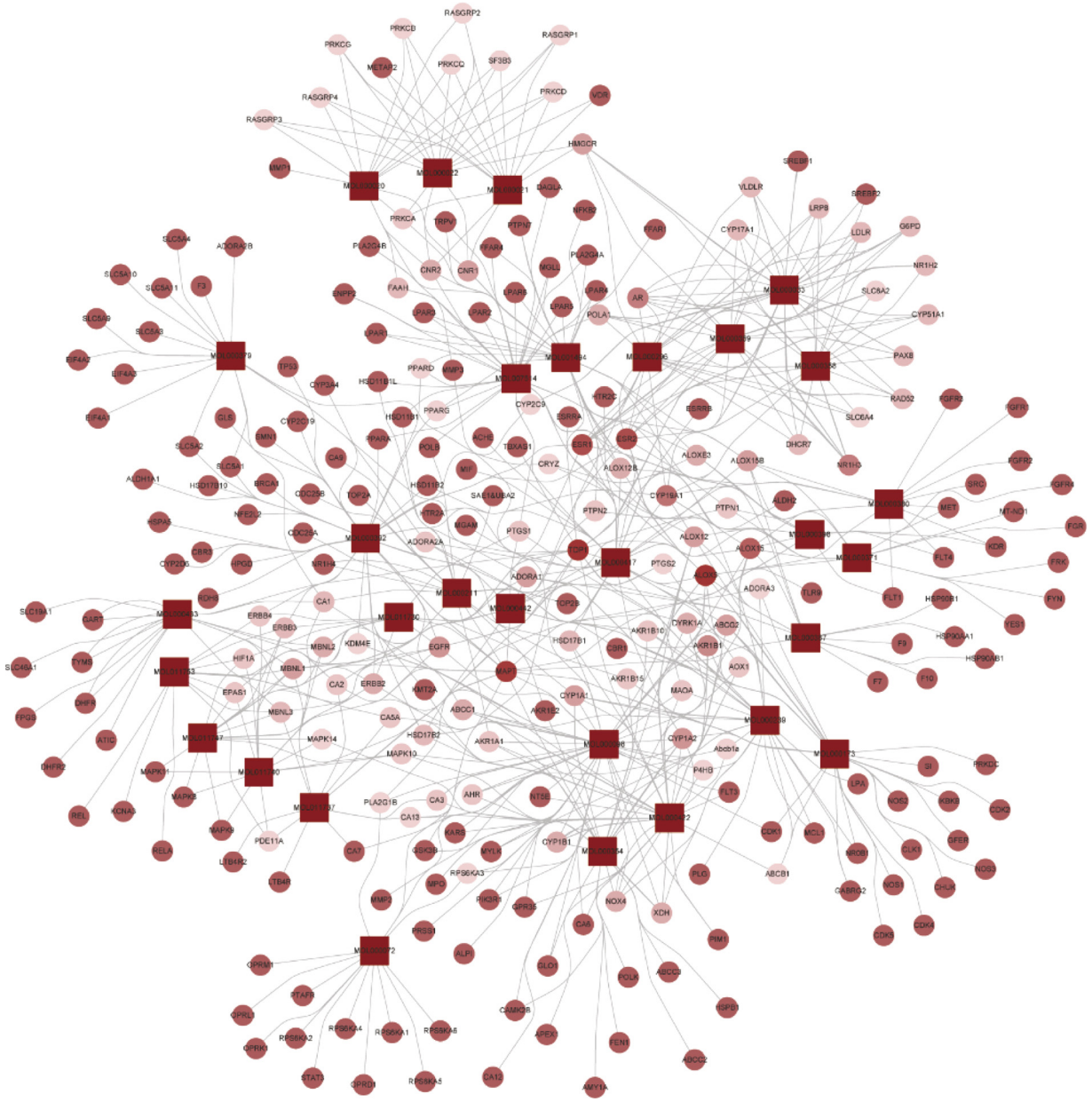

Fig. 1. "Ingredient-target" network of Yupingfengsan. The rectangle represent ingredients, and the ellipse represent putative targets.

2.3. Enrichment analysis

Overlapped between Yupingfengsan targets and LAD DEGs were extracted. Subsequently, for the biological and functional annotation of the overlapped genes, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed by gene set enrichment analysis (GSEA) (version 3.0, http://software.broadinstitute.org/gsea/), a computational method that determines whether defined set of genes shows statistically significant [39,40]. The false discovery rate (FDR) below 0.05, the smaller the more enriched, was considered to be the significant biological process and pathway.

2.4. Protein-protein interaction (PPI) and network construction

To analyze Protein-protein interaction between overlapped DEGs at the protein level, Overlapped DEGs were input into the Search Tool for the Retrieval of Interacting Genes (STRING) database [41] (Version 10.5, https://string-db.org/), the species limited to "Homo sapiens" and medium confidence score > 0.4 was considered significant.

"Ingredients-targets network" and "PPI network" were created via utilizing the network visualization software Cytoscape [42](version 3.7.0, http://www.cytoscape.org/), the network was analyzed using topological parameters, subnetwork was extracted according to greater than average degree. The Molecular Complex Detection (MCODE) plugin was used for searching the most significant module from the network [43]. MCODE criteria for selection were as follows [44]: MCODE scores ≥ 5, Degree cutoff = 2, Node score cutoff = 0.2, K-core = 2 and Max depth = 100.

2.5. Prediction and verification of key target genes

To putative key target genes of Yupingfengsan in the treatment of LAD, core genes from PPI network and genes from the main pathway were overlapped. For further study, paired samples were screened from TCGA database to verify the mRNA expression of these candidates

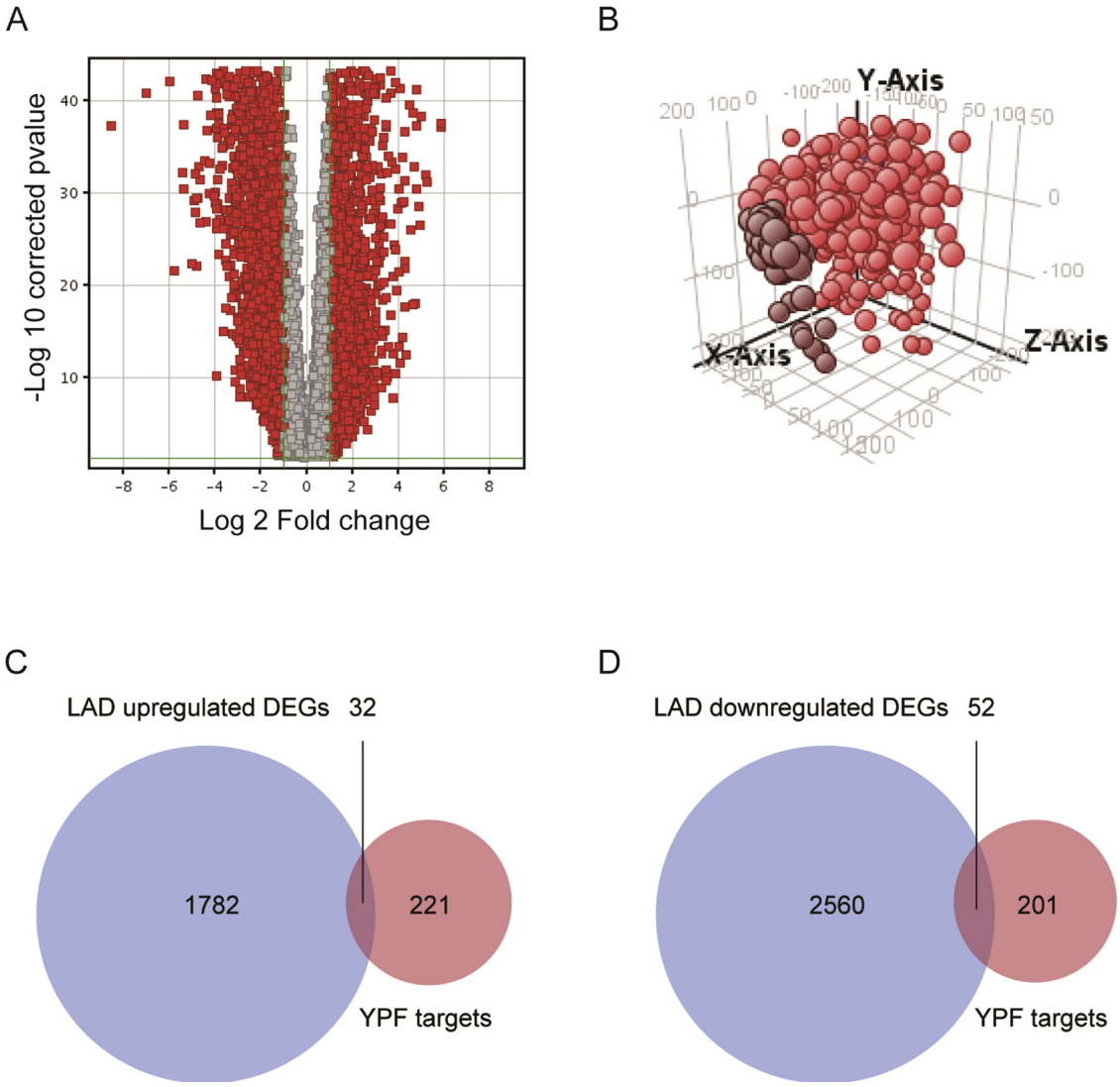

Fig. 2. Differential expression genes (DEGs) of lung adenocarcinoma (LAD). (A) Volcano plot of 4426 DEGs. (B) 3D PCA quality control plot, Red nodes represent LAD samples and brown nodes represent adjacent normal samples. Overlapped genes between Yupingfengsan (YPF) putative targets and 1814 upregulated (C) and 2612 downregulated (D) DEGs in LAD.

between LAD and adjacent normal tissues, mRNA profile data and paired samples of LAD were matched with VLOOKUP index in excel. Finally, differential expression of candidates between LAD and paired adjacent normal tissue was calculated.

2.6. Survival analysis

To investigate the prognostic significance of key target of Yupingfengsan in treatment LAD, Kaplan-Meier plotter (http://kmplot.com), an online database which includes both clinical and expression data, was utilized to verify the relationship between candidates and prognosis of LAD [45].

2.7. Statistical analysis

Differences of gene expression between the individual groups were analyzed using *t*-test by Graphpad Prism 7 (GraphPad Software, CA, USA). A two-tailed $P < 0.05$ were considered significant for all tests.

3. Results

3.1. Identification of Yupingfengsan chemical ingredients

Yupingfengsan contains 3 herbs, Huang Qi, Bai Zhu and Fang Feng. For each of these herbs, the ingredients with OB ≥ 30%, DL ≥ 0.18 and DL ≥ 4 h were selected from TCMSP database. Thus, a total of 30 ingredients were ultimately chosen for further investigation, the ADME information of these ingredients was shown in Table 1.

3.2. Putative targets of Yupingfengsan

We collected 465 targets from SwissTargetPrediction and 287 targets from SuperPred, the targets were merged and the repeated genes were removed. A total of 253 putative targets were identified, as shown in table S1.

3.3. Ingredient-targets network construction

Cytoscape software was used to visualize ingredient-targets network, the network included 283 nodes and 725 edges, as shown in Fig. 1.

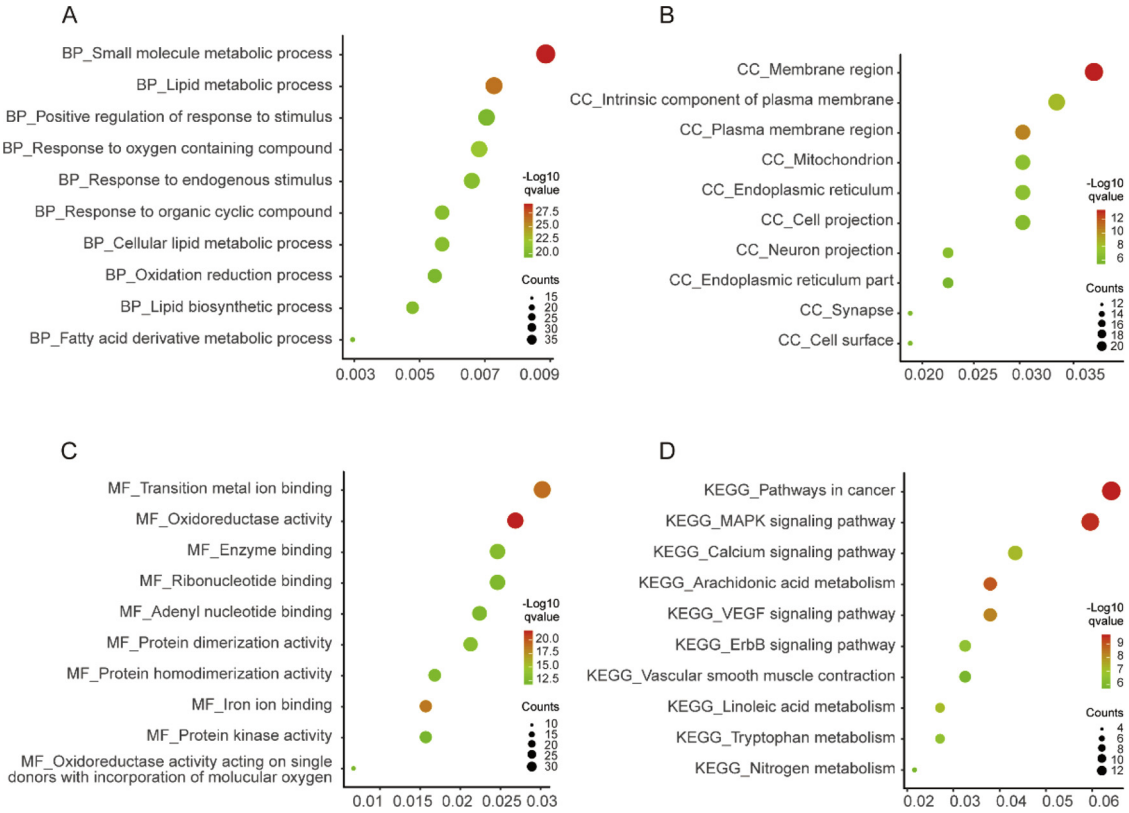

Fig. 3. Enrichment analysis of overlapped genes, Top ten enrichment results were exhibited, respectively. (A) Biological process (BP). (B) Cellular components (CC). (C) Molecular function (MF) and (D) KEGG pathway.

3.4. Identification DEGs of LAD

Totally 4426 DEGs were identified in TCGA LAD database (Fig. 2A-B), including 1814 upregulated and 2612 downregulated genes.

3.5. Overlapped between Yupingfengsan and DEGs of LAD

Using venn diagram, A total of 84 overlapped genes were obtained between Yupingfengsan putative targets and DEGs of LAD, include 32 upregulated (Fig. 2C) and 52 downregulated genes (Fig. 2D).

3.6. Enrichment analysis

To classify the biological function of overlapped genes, GO and KEGG pathway enrichment analysis was performed using GSEA. GO analysis revealed that 84 overlapped genes were significantly enriched in biological process (BP, Fig. 3A), including small molecule metabolic process, lipid metabolic process, response to oxygen containing compound, cellular lipid metabolic process, response to organic cyclic compound, response to endogenous stimulus, lipid biosynthetic process, oxidation reduction process, positive regulation of response to stimulus and fatty acid derivative metabolic process. Cellular component (CC, Fig. 3B) analysis showed that membrane region, plasma membrane region, intrinsic component of plasma membrane, endoplasmic reticulum, mitochondrion, neuron projection, cell projection, synapse, cell surface, and endoplasmic reticulum part were mostly classification. In term of molecular functions (MF, Fig. 3C), the overlapped genes were mainly associated with oxidoreductase activity, transition metal ion binding, iron ion binding, protein dimerization activity, enzyme binding, ribonucleotide binding, adenyl nucleotide binding, protein homodimerization activity, oxidoreductase activity acting on single donors with incorporation of molecular oxygen and protein kinase activity. In addition, the most enrichment terms of KEGG pathway (Fig. 3D) included pathways in cancer, MAPK signaling pathway, arachidonic acid metabolism, VEGF signaling pathway, calcium signaling pathway, linoleic acid metabolism, tryptophan metabolism, ErbB signaling pathway, vascular smooth muscle contraction and nitrogen metabolism.

3.7. PPI network construction and subnetwork extraction

A total of 84 overlapped genes were input into String online tool for proteins interaction analysis. PPI network was construct using Cytoscape software, as shown in Fig. 4A. The network included 77 nodes and 214 edges. Subnetwork, contained 33 nodes and 117 edges, was extracted if degree greater than 5.558 (average degree). 33 nodes as core genes for further investigation. Subsequently, we utilized MCODE plugin to analyze the whole network, 4 modules were chosen (Fig. 4B, Module 1, Module 2, Module 3 and Module 4). Among them, module 1 showed the most significantly scores.

3.8. Putative key targets of Yupingfengsan in treating LAD

Potential target genes of Yupingfengsan on LAD were predicted, 33 core genes from network and 16 genes from main pathway were overlapped, 6 candidates, included PRKCB, PIK3R1, KDR, PLA2G1B, PLA2G4A and PTGS2 were screened. Finally, A total of 58 paired samples of LAD and adjacent normal tissue were collected from TCGA database to verify the mRNA differential expression of these 6 genes, as shown in Fig. 5A-F, compared with paired adjacent normal tissue, PLA2G4A was upregulated and 5 candidates including PRKCB, PIK3R1, KDR, PLA2G1B and PTGS2, were downregulated among LAD samples significantly (all $P < 0.001$).

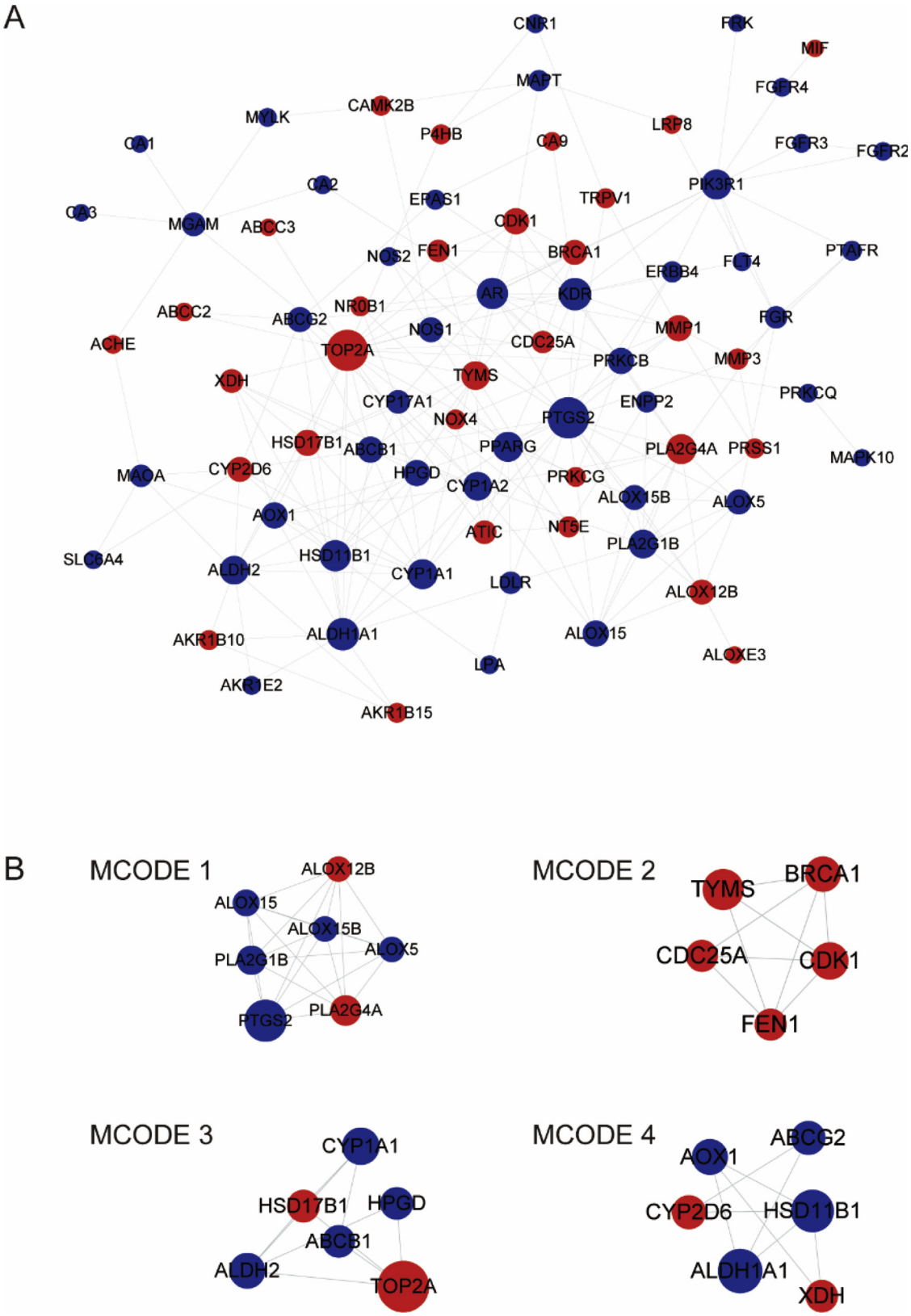

Fig. 4. Protein interaction (PPI) network construction. (A) PPI network, the red nodes represent upregulated genes, blue nodes represent downregulated genes. (B). 4 modules were extracted by MCODE plugin.

3.9. Survival analysis

Kaplan-Meier plotter was utilized to evaluate the prognostic value of these 6 key targets of Yupingfengsan in treatment LAD. Among them, High expression mRNA of 4 genes, PRKCB, KDR, PIK3R1, PLA2G1B, predicted a longer overall survival (OS, all Log rank $P < 0.01$, Fig. 6A-D) and favorable progression-free survival (FPS, all Log rank $P < 0.001$, Fig. 6E-H). In terms of PLA2G4A and PTGS2, only was

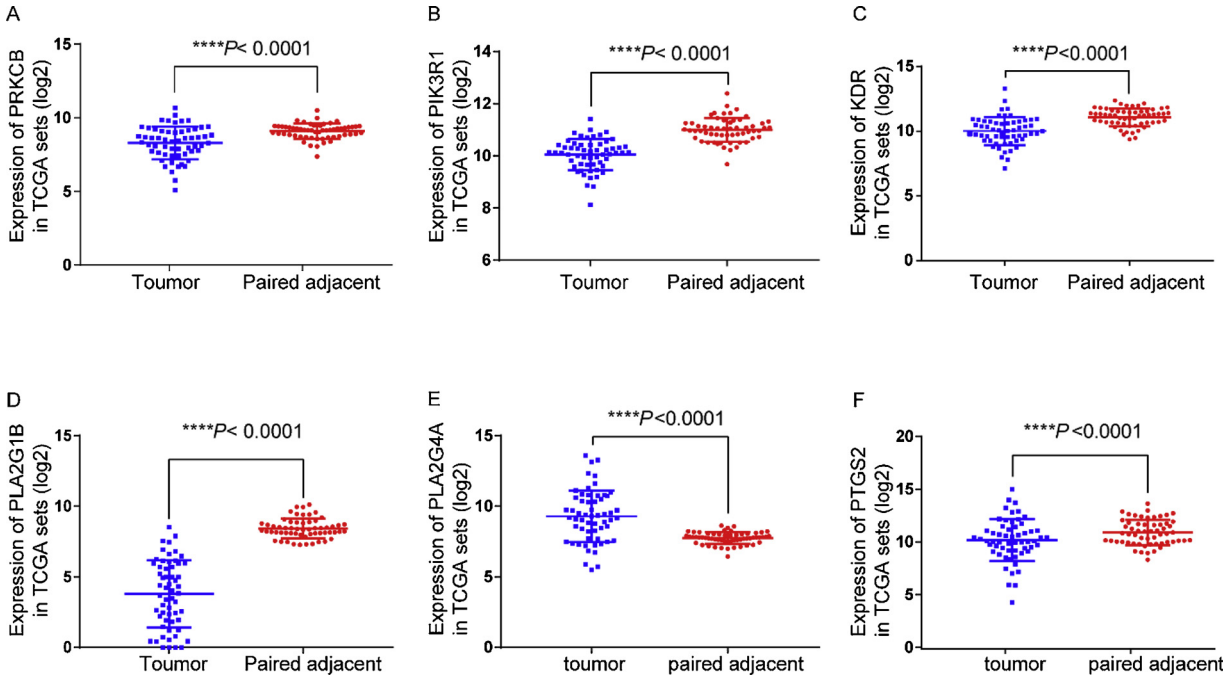

Fig. 5. mRNA differential expression between LAD and paired adjacent normal tissues based on TCGA database. (A) PRKCB. (B) PIK3R1. (C) KDR. (D) PLA2G1B. (E) PLA2G4A and (F) PTGS2.

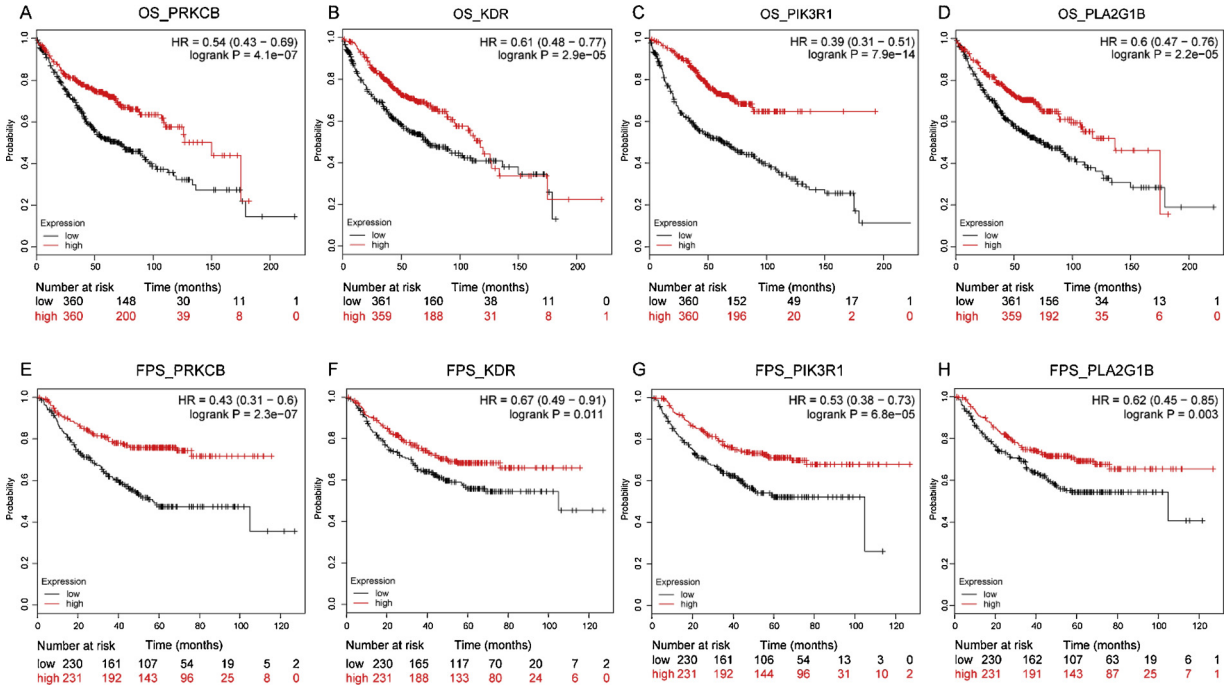

Fig. 6. Associations between survival times. OS (A) PRKCB, (B) PIK3R1, (C) KDR, (D) PLA2G1B. FPS (E) PRKCB. (F) PIK3R1. (G) KDR. (H) PLA2G1B in LAD based on Kaplan-Meier plotter database. The red lines represent patients with high mRNA expression, black lines represent patients with a low mRNA expression.

observed have a relationship with OS, unfortunately, the same trends was not verified with FPS (Log rank $P = 0.91$ and Log rank $P = 0.32$, respectively).

Finally, the most probably mechanism of Yupingfengsan in treatment LAD was drawn, as shown in Fig. 7.

4. Discussion

Network pharmacology provides a powerful tool for exploring the compatibility and mechanisms of traditional Chinese formulae. In the present study, we integrated network pharmacology and bioinformatics analysis to predict the potential targets and probable mechanism of Yupingfengsan in treatment LAD. Firstly, a total of 253 targets from 30 active ingredients of Yupingfengsan were predicted. 4426 DEGs were identified from TCGA database. 84 overlapped genes were obtained (32 upregulated, 52 downregulated) between Yupingfengsan putative targets and DEGs of LAD. Secondly, enrichment analysis was performed, the results revealed that MAPK, VEGF and ErbB signaling pathways seemed to be the most likely mechanism. Subsequently, 84 overlapped genes were used to construct PPI network, 33 core genes were extracted

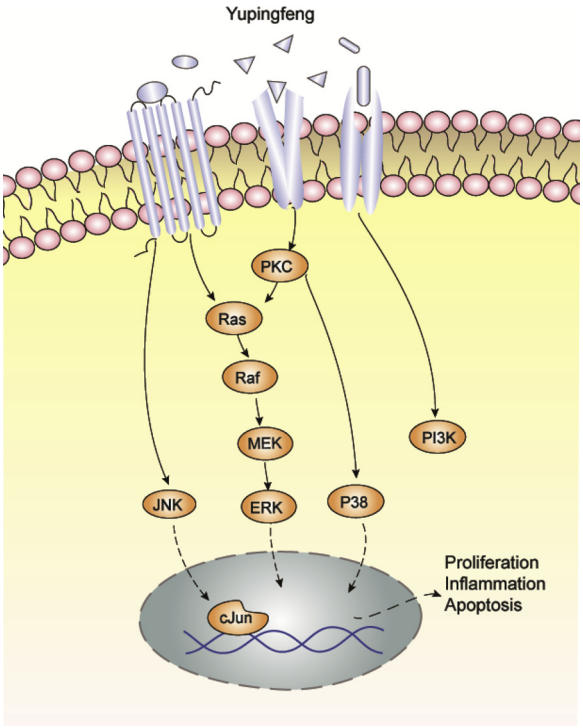

Fig. 7. Mechanism diagram of Yupingfengsan in treatment LAD.

by using a network topological analysis. And then, 6 key target genes of Yupingfengsan in treatment LAD were screened, including PLA2G4A, PRKCB, PIK3R1, KDR, PLA2G1B and PTGS2. Finally, based on TCGA database, 6 candidates were verified significantly different expression between LAD and paired adjacent normal tissues. Survival analysis indicated that 4 candidates, PRKCB, PIK3R1, KDR, PLA2G1B, suggest a significant correlation with LAD prognosis.

To summarize previous publications. Yupingfengsan was used as an anti-inflammatory and immune-regulatory agent [46]. On the one hand, the possible mechanism by which Yupingfengsan reducing inflammatory response has been proved act on following aspects, attenuated inflammatory cell infiltration, reduced the production and release of inflammatory factors, including TNF-α, IL-6, IL-4, IL-1β [47]. Regulated the expression of inflammatory mediators [48,49] NLRP3, HMGB1, TGF-β1, and TLR4/NF-κB signaling pathway [50]. On the other hand, in terms of regulating the immune function, Yupingfengsan could not only raise thymus gland and spleen index, increase the phagocytosis of the macrophage, improve a proportion of CD4 + /CD8 + and NK cells' activity [51–53], but also decrease the levels of Foxp3(+) Treg [54], JAK/STAT signaling pathway participated in an immune-regulating process [55].

As we know, avoiding immune destruction, tumor promotion inflammation, and genome instability are hallmarks of cancer [56]. The above research provided powerful evidence in the aspect of Yupingfengsan anti-inflammatory and immune-regulation in lung cancer. However, from the perspective of functional genomic research, the mechanism of Yupingfengsan in treatment of lung cancer remains unclear. The relationship between Yupingfengsan and the core functional genomic and pathway that refer in our results has not been confirmed from the previous publications. Our findings provided a novel insight for the effects of Yupingfengsan on LAD.

There are several limitations to this study. First, this finding relied on integrating network pharmacology and bioinformatics analysis. The predicted targets were influenced by different methods of identifying targets. Second, cancer is a result of highly complex molecular mechanisms, further experimental studies are necessary to clarify predicted targets and related signaling pathways of Yupingfengsan for LAD.

5. Conclusion

Differentially expressed in tumors, PLA2G4A, PRKCB, PIK3R1, KDR, PLA2G1B and PTGS2 are potential therapeutic targets of Yupingfengsan in treatment LAD, and MAPK, VEGF and ErbB signaling pathways are involved.

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Contributors statement

LL conceptualized and the article. LS conducted the data download and analysis. WC and BZ performed data extraction and consolidation. YC participated in drafting and writing.

Declaration of Competing Interest

None.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eujim.2019.100967.

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