Network pharmacology analysis and clinical verification of *Panax notoginseng* saponins in deep venous thrombosis prevention

BIN YAN[1*], YACHAN NING[1*], JULONG GUO[2*], LIMIN LIU[3] and CHUNMEI WANG[1]

[1]Department of Intensive Care Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100053, P.R. China;
[2]Department of Vascular Surgery, Xuanwu Hospital, Capital Medical University, Beijing 100053, P.R. China;
[3]Department of Orthopaedics, Xuanwu Hospital, Capital Medical University, Beijing 100053, P.R. China

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Abstract. In the present study, the mechanism of *Panax notoginseng* saponins (PNS), the extract of *Panax notoginseng*, against deep vein thrombosis (DVT) was explored by networks pharmacology and its effect was demonstrated through clinical data. PNS includes 5 main active components, which have 101 targets. A total of 1,342 DVT-related targets were obtained, 55 of which were the common targets of PNS and DVT. AKT1, TNF, IL1B, EGFR, VEGFA and MAPK3 were selected as hub genes from the protein-protein interaction network. The potential anti-DVT mechanism of PNS may involve the AGE-RAGE signaling pathway and the PI3K-Akt signaling pathway. Molecular docking presented a total of 10 binding interactions, with all molecules showing good binding ability with PNS-DVT common hub target genes (all binding energy <-6 kcal/mol). Analysis of clinical data showed that the combined use of PNS significantly reduced the incidence of postoperative DVT in patients undergoing orthopedic surgery compared with the use of low-molecular-weight heparin alone, which is the most commonly used clinical anticoagulant.

Introduction

Deep venous thrombosis (DVT) can develop into pulmonary thromboembolism (PTE), which may even be life-threatening. DVT and PTE, collectively known as venous thromboembolism (VTE), after acute coronary syndrome and stroke, is the third most common clinical cardiovascular disease, and is also one of the preventable causes of death in hospitalized patients (1-3). The incidence of DVT is high in surgical patients, with hospital-acquired DVT occurring in up to 60% after major orthopedic surgery (4). Therefore, the prevention of DVT becomes essential. Prevention with drugs is one of the most effective measures to reduce the risk of DVT formation. Widely used anticoagulants include warfarin, heparin, low-molecular-weight heparin (LMWH) and novel oral anticoagulants (5). However, even with the use of anticoagulant drugs, the incidence of DVT in patients with surgery and trauma cannot be ignored (6,7). In addition, the adverse reactions of anticoagulant drugs should be noted, such as hemorrhage, hematoma and thrombocytopenia (8,9). New treatments are urgently needed to prevent DVT.

Traditional Chinese medicine (TCM) and herbal medicines have been widely used for thousands of years to treat various diseases, including thrombosis. TCM is mainly derived from natural plants and has the advantages of good efficacy, less toxicity and side effects, and low cost (10). *Panax notoginseng* is a classical TCM rich in >70 kinds of *Panax notoginseng* saponins (PNS), mainly including notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and ginsenoside Rd qt (7,11,12). PNS have been found to have positive effects on various diseases, including coronary heart disease, ischemic stroke, gastrointestinal injury and Alzheimer's disease (11-14). The mechanisms involved in these effects include anti-inflammation, anti-oxidation, inhibition of platelet aggregation, anti-apoptosis, promotion of blood circulation, improvement of vascular endothelial function and regulation of blood lipids (12,15,16). TCM indicates that *Panax notoginseng* may be used for the prevention and treatment of thrombosis. However, the mechanisms underlying the effect of PNS against DVT have remained to be fully elucidated.

In recent years, an increasing number of studies have been devoted to exploring the mechanisms of action of TCM in the treatment of various diseases based on network pharmacology (10). Network pharmacology combines pharmacology and bioinformatics to reveal the specific targets of drug interventions in the processes of disease, which is helpful to promote the development of precision medicine (11,14). In the present study, several public databases were used to predict PNS-DVT targets and establish pharmacological networks, from which key drug components and hub targets

Correspondence to: Professor Chunmei Wang, Department of Intensive Care Medicine, Xuanwu Hospital, Capital Medical University, 45 Changchun Street, Xicheng, Beijing 100053, P.R. China
E-mail: drwangchunmei@sina.com

*Contributed equally

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were screened. Subsequently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis combined with molecular docking verification were performed to investigate the complex effects of PNS in the prevention of DVT. Finally, clinical data were used to confirm the efficacy and safety of PNS in preventing DVT.

Materials and methods

Prediction of the targets of PNS. The main active components of PNS were identified from the TCM Systems Pharmacology Database and Analysis Platform (TCMSP; http://lsp.nwu.edu.cn/tcmsp.php), which is a unique systems pharmacology platform of Chinese herbal medicines (17). Symmap v2 (http://www.symmap.org/) was used to identify targets related to these main active components in the study. Symmap provides massive information on herbs/ingredients, targets, the clinical symptoms and diseases, as well as the associations among them (18).

Collection of PNS-DVT common targets. The Online Mendelian Inheritance in Man (OMIM; https://omim.org/) (19), DrugBank (https://go.drugbank.com/) (20), GeneCards (https://www.genecards.org/) (21) and DisGeNET (https://www.disgenet.org/) (22) databases were searched for genes associated with DVT using 'deep venous thrombosis' as the keyword. Common PNS-DVT targets were obtained by drawing a Venn diagram of DVT-related genes and targets related to the active components of PNS using an online drawing tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Cytoscape software (v3.9.0) was used to visualize the herb-component-target gene network (23). The Degree value of each active compound was calculated using Cytoscape's application named Cytohubba (v0.1) (24).

Construction of protein-protein interaction (PPI) network. The PNS-DVT common targets were uploaded to the Search Tool for the Retrieval of Interacting Genes (STRING; v.11.5; https://cn.string-db.org/) database to obtain the PPIs (25). '*Homo sapiens*' and 'interaction score ≥0.7' were used as the screening criteria. Cytoscape software (v.3.9.0) was used to visualize the PPI network (23). The degree value of each node was also calculated using Cytohubba for screening hub genes (24).

Enrichment analysis. To determine the functions and signaling pathways involved in the PNS-DVT common targets, GO and KEGG pathway enrichment analyses were performed using the Metascape (https://metascape.org) platform (26). After uploading PNS-DVT common targets to Metascape, GO terms in the categories biological process (BP), cellular component (CC) and molecular function (MF), and KEGG pathways were obtained from the enrichment analysis. The conditions for the analysis were set as min overlap=3, P-value cutoff=0.01 and min enrichment=1.5. The results of the enrichment analysis were visualized using GraphPad Prism 9.0.0 (Dotmatics).

Molecular docking. Molecular docking was used to examine receptor-ligand interactions and affinities. The PDB files of the 3D structure of the proteins expressed by the hub genes were downloaded from the Protein Data Bank (PDB) database (https://www.rcsb.org/) (27), and PyMol (v.2.6) software was used to remove water molecules and unrelated ligands from the 3D structure. The PDB file of the protein was then imported into AutodockTools (v.1.5.7) (28) software for hydrogenating and saved in PDBQT format. Mol2 files of hub active compounds were downloaded from the TCMSP database and then saved in PDBQT format after hydrogenating and rotatable bonds setting by AutodockTools (v.1.5.7) software. Finally, by setting the maximum 'Gird box', i.e., the blind docking method, the molecular docking was verified in AutodockTools (v.1.5.7) software, and the results with the lowest binding energy were visualized by PyMol (v2.6) software (29). A binding energy <0 kcal/mol indicates that the ligand can spontaneously bind to the protein.

Validation of clinical data. To verify the efficacy of PNS in preventing DVT, patients undergoing orthopedic surgery at the Department of Surgery of Xuanwu Hospital, Capital Medical University (Beijing, China) from January 2016 to December 2018 were screened. The inclusion criteria were as follows: Age ≥18 years; Caprini scale scores suggest ≥ moderate VTE risk; the anticoagulant therapy received during the perioperative period was LMWH (hypodermic injection; 4,000-8,000 AxaIU once daily; 100 AxaIU/kg) or LMWH plus PNS (drug named Xue-Shuan-Tong oral tablets; 100 mg; 3 times daily) (7); deep vein ultrasound of the lower extremities on admission did not reveal DVT, and the lower extremity deep vein ultrasound was reexamined before discharge. Pregnant women, patients with coagulopathy and/or contraindications to anticoagulation, and patients already on anticoagulants prior to hospitalization, were not included. This study was approved by the Institutional Review Board of Xuanwu Hospital, Capital Medical University (Beijing, China; approval no. [2017]088). The primary endpoint was the incidence of DVT in a lower extremity. Other endpoints included the incidence of major bleeding (hemoglobin lost ≥2 g/l), pulmonary embolism and pulmonary embolism-related death.

Statistical analysis. Descriptions of statistical methods relevant to networks pharmacology were presented in preceding each part of the *Methods* section. In the validation of clinical data, normally distributed continuous variables were analyzed using a two-sided unpaired t-test, while non-normally distributed variables were analyzed using the Wilcoxon rank-sum test. Dichotomous data were compared using Fisher's exact test or chi-square test. Significance was defined as $P<0.05$.

Results

Predicted targets of PNS. Notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and ginsenoside Rd qt were the 5 main active components in PNS (11,12), and their details are described in Table I. Among them, ginsenoside Rd qt has the best oral bioavailability and drug-likeness quality, so it may be the main bioactive component in oral management. The results obtained from the Symmap v2 databases were integrated to obtain the 101 non-repetitive targets with the five active PNS compounds (Fig. 1A). As shown in Fig. 2,

Table I. Active components of *Panax notoginseng* saponins.

Molecule ID	Compound name	OB%	DL
MOL007487	Notoginsenoside R1	5.43	0.13
MOL005341	Ginsenoside Rg1	10.04	0.28
MOL005338	Ginsenoside Re	4.27	0.12
MOL007476	Ginsenoside Rb1	6.29	0.04
MOL007480	Ginsenoside Rd qt	12.23	0.77

OB, oral availability; DL, drug-likeness.

PNS mainly acts through multiple components corresponding to multiple targets.

Collected common targets of PNS-DVT. After deduplication, a total of 1,342 DVT-related targets were obtained from the OMIM, DrugBank, GeneCards and DisGeNET databases (Fig. 1B). There were 55 common targets shared by PNS active components and DVT (Fig. 1C).

Construction of the PPI network. A total of 55 common targets of PNS and DVT were introduced into the STRING database for PPI network analysis. As presented in Fig. 3A, there were 52 nodes (genes) with 301 edges (interactions) in the PPI network, and those nodes with higher degree values were regarded as hub target genes. AKT1, TNF, IL1B, EGFR, VEGFA and MAPK3 were the top 6 targets, with degree values of 32, 28, 25, 25, 25 and 23, respectively. The network of the 5 main active compounds and hub target genes is shown in Fig. 3B.

GO and KEGG pathway enrichment analyses. The Metascape platform was used for the GO and KEGG pathway analyses, and the top 10 terms in each category were visualized (Fig. 4). The top 3 enriched terms in GO BP were 'positive regulation of cell migration', 'positive regulation of cell motility' and 'positive regulation of locomotion'. The top 3 enriched terms in GO CC were 'platelet alpha granule lumen', 'vesicle lumen' and 'platelet alpha granule'. The top 3 enriched terms in GO MF were 'signaling receptor regulator activity', 'signaling receptor activator activity' and 'cytokine receptor binding'. The 'advanced glycation end products (AGE)/receptors for AGE (RAGE) signaling pathway in diabetic complications', 'pathways in cancer' and the 'PI3K-Akt signaling pathway' were the most significant KEGG signaling pathways.

Molecular docking. According to the targeting relationship shown in Fig. 3B, the 6 hub target genes from the PPI network were docked to the 5 active compounds of PNS. Table II shows the PDB ID of the hub target genes used in molecular docking. The results presented a total of 10 binding interactions, with all molecules showing a binding energy <-6 kcal/mol with the targets. In other words, these five active PNS compounds have good binding ability with PNS-DVT common hub target genes. Both notoginsenoside R1 and ginsenoside Rb1 had 3 targets. The visual docking results are shown in Fig. 5.

Clinical results of PNS in preventing DVT. A total of 194 patients were screened for this clinical validation, and 99 and 95 patients were in the LMWH group and PNS + LMWH group, respectively. Female patients account for 70.71 and 70.53% in the two groups, with average ages of 69.83 and 68.61 years, respectively. As shown as in Table III, there were no significant differences in baseline characteristics between the two groups. The incidence of postoperative DVT in the LMWH group was 25.25%, which was significantly higher than the 12.63% in the LMWH + PNS group (P=0.025), while there were no significant differences in coagulation function indexes except D-dimer (P=0.044) after surgery between the two groups. No major bleeding, pulmonary embolism or pulmonary embolism-related death occurred in either group.

Discussion

The clinical data showed that the combined use of PNS significantly improved the effect of LMWH in preventing DVT after orthopedic surgery. The molecular mechanism of this effect was explored by network pharmacology analyses. In this study, it was indicated that PNS and DVT have 55 common gene targets. Further analysis showed that PNS may use AKT1, TNF, IL1B, EGFR, VEGFA and MAPK3 as hub targets to prevent DVT.

AKT1, also known as protein kinase B, is a potent signal transducer of multiple signaling functions in platelets. Studies have confirmed that AKT is involved in the positive regulation of megakaryopoiesis and thrombopoiesis (30,31). As the critical role of GPIb-IX-mediated early signals, AKT mediates a variety of agonists-induced signaling cascades in platelets. Under the stimulation by agonists, rapidly activating AKT is involved in multiple signaling pathways, such as the PI3K/Akt signaling pathway, contributing to integrin activation, thromboxane synthesis and degranulation (32-34). Inflammation plays an important role in the formation of DVT (35). Inflammatory cells, particularly macrophages, are the sources of the proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) (36). However, the connection between the TNF-α and thrombosis remains controversial. TNF-α can induce tissue factor (TF), which is the key initiator of the physiological coagulation process through activating the exogenous coagulation pathway (37). As a strong stimulator, TNF-α can activate the coagulation system, which is manifested as the downregulation of physiological anticoagulant mechanisms and the inhibition of fibrinolysis (38). However, it has also been proposed that an altered inflammatory cytokine profile may be the result of venous thrombosis rather than a cause of it (39). Of note, a study has found that TNF-α interacting with TNF-Rp55 enhances the resolution of venous thrombosis through the increased expression of fibrinolytic mediators and enzymes linked to collagen remodeling by macrophages (40).

IL-1β is produced by macrophages and monocytes and is a marker of an early inflammatory response. IL-1β binds to the IL-1β receptor on endothelial cells and activates the NF-κB pathway to cause endothelial injury, and activates TF, coagulation and von Willebrand factor to promote platelet adhesion and fibrin deposition, thereby initiating and aggravating thrombosis (41-43). Epidermal growth factor receptor (EGFR) is a widely used prognostic marker

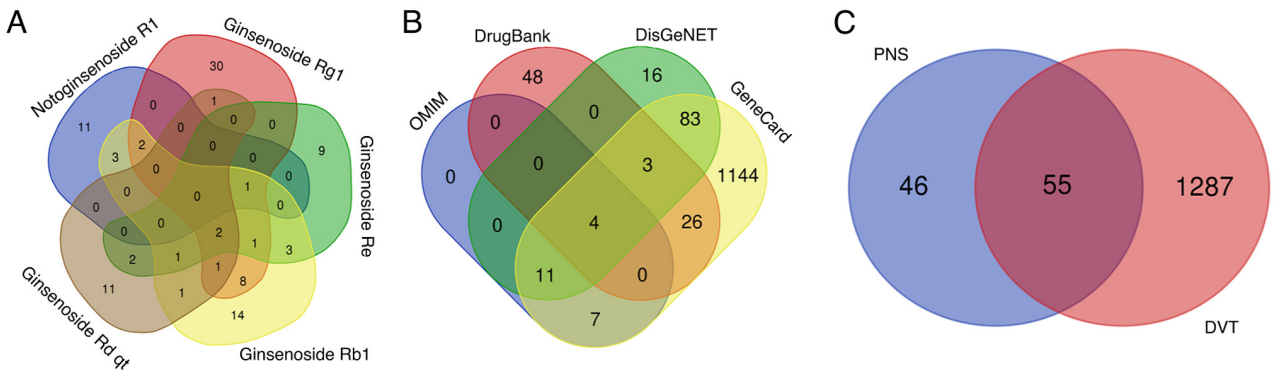

Figure 1. Venn diagrams. (A) Targets of PNS compounds. (B) DVT-related genes. (C) Common targets of PNS and DVT. PNS, *Panax notoginseng* saponins; DVT, deep vein thrombosis.

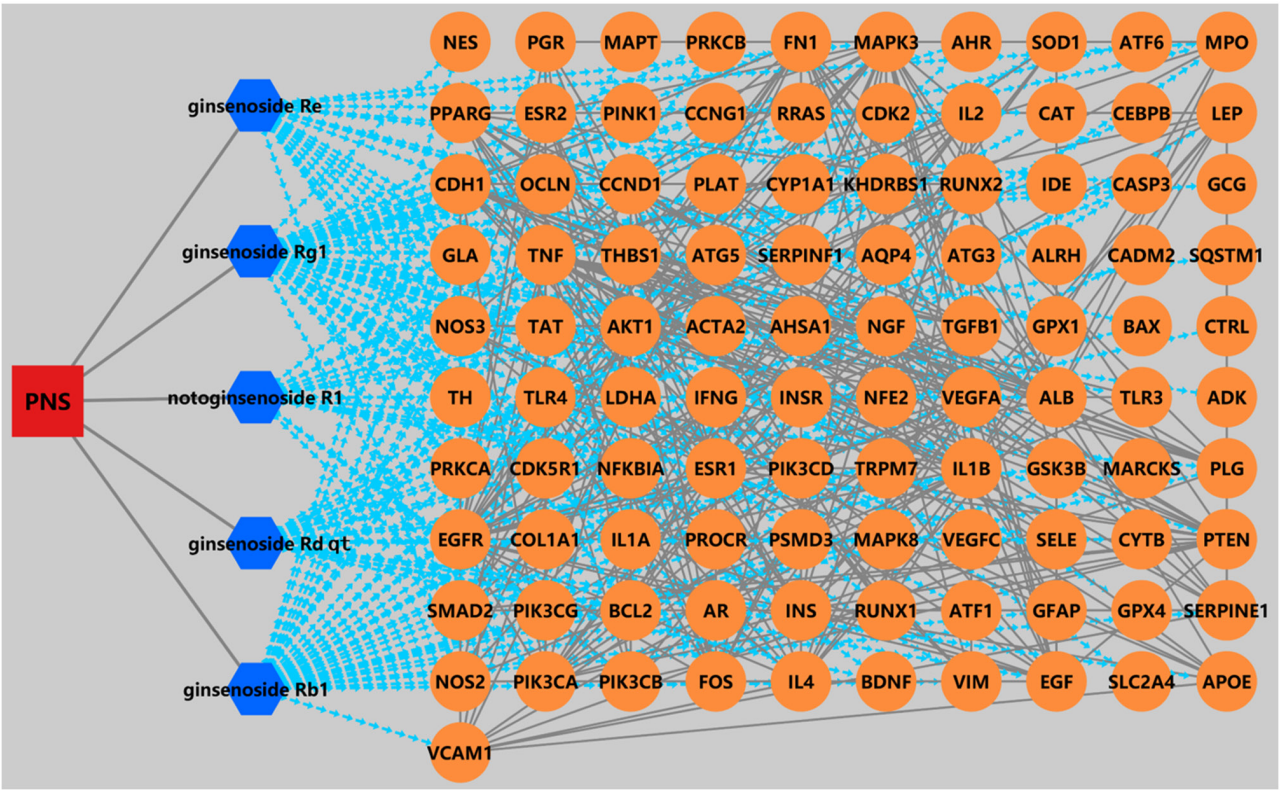

Figure 2. PNS-main active components-common targets network. PNS, *Panax notoginseng* saponins.

for numerous cancers, and increasing studies have found an association between EGFR and the occurrence of DVT and other thromboembolic complications, particularly in cancer patients. EGFR can stimulate the production of growth factors, including vascular endothelial growth factor (VEGF), while VEGF is a chemokine factor for cells that expresses TF, which is involved in thrombosis, as mentioned above (44-46). As a member of the MAPK family, MAPK3, also known as extracellular signal-regulated kinase 1 (ERK1), is associated with thrombin-activated platelet aggregation. Activated ERK is important in GPIb-IX-mediated signaling, leading to integrin activation and, thus, integrin-dependent stable platelet adhesion, aggregation and thrombosis (33,47).

The mechanism of the above hub genes in thrombosis was closely related to the results revealed by GO enrichment analysis. KEGG enrichment analysis indicated that the common targets were mainly enriched in the 'AGE-RAGE signaling pathway in diabetic complications', 'pathways in cancer' and the 'PI3K-Akt signaling pathway'. AGEs and RAGEs were first studied in diabetes (48). The AGE/RAGE pathway has been substantiated to be involved in oxidative stress, inflammation and a variety of diseases, including cardiovascular diseases and thrombosis (49). RAGE inhibition can suppress the release of proinflammatory cytokines IL-6, IL-1β and TNF-α (48). In addition, RAGE inhibition markedly suppressed malondialdehyde and reactive oxygen species levels and increased the level of the antioxidant substance superoxide dismutase, which effectively alleviated AGE-induced oxidative stress. Inhibition of the AGE/RAGE axis also significantly increased levels of nitric oxide-suppressed endothenin-1

Table II. Molecular docking results between the hub targets and main compounds.

Hub gene	PDB ID	Compound name	Binding energy, kcal/mol
AKT1	7myx	Notoginsenoside R1	-6.7
TNF	5uui	Notoginsenoside R1	-7
TNF	5uui	Ginsenoside Rg1	-6.4
TNF	5uui	Ginsenoside Rb1	-6.7
IL1B	AF_P01584_F1	Ginsenoside Rg1	-7.1
IL1B	AF_P01584_F1	Ginsenoside Rb1	-8.2
EGFR	5ug9	Ginsenoside Rd qt	-9.1
VEGFA	1mkk	Notoginsenoside R1	-7.3
VEGFA	1mkk	Ginsenoside Rb1	-7.2
MAPK3	6ges	Ginsenoside Re	-8.7

PDB, protein databank.

Table III. Demographic and clinical characteristics of participants.

Variable	LMWH alone (n=99)	LMWH + PNS (n=95)	P-value
Female sex	70 (70.71)	67 (70.53)	0.978
Age, years	69.83±9.23	68.61±11.12	0.578
Body mass index, kg/m^2	25.72±3.98	25.64±4.12	0.887
Hypertension	55 (55.56)	46 (48.42)	0.320
Diabetes	33 (33.33)	26 (27.37)	0.367
Location of surgery			0.811
Knee	62 (62.63)	54 (56.84)	
Hip	35 (35.35)	38 (40.00)	
Calcaneus	1 (1.01)	1 (1.05)	
Ankle	1 (1.01)	2 (2.11)	
Pre-operation			
PT, sec	13.20±0.72	13.32±0.83	0.282
TT, sec	16.12±1.11	16.77±7.90	0.419
APTT, sec	37.33±5.09	36.91±4.63	0.553
INR	1.01±0.07	1.02±0.08	0.441
Fibrinogen, g/l	3.63±0.96	3.56±0.79	0.546
D-dimer, μg/ml	0.61 (0.35, 1.70)	0.69 (0.34, 1.93)	0.999
Post-operation			
PT, sec	13.82±0.86	13.66±0.85	0.214
TT, sec	15.04±1.65	14.76±0.92	0.176
APTT, sec	42.88±6.86	42.39±6.40	0.638
INR	1.07±0.09	1.05±0.08	0.109
Fibrinogen, g/l	4.64±1.32	4.95±1.31	0.140
D-dimer, μg/ml	1.78 (1.05, 2.76)	1.34 (0.87, 2.32)	0.044
DVT	25 (25.25)	12 (12.63)	0.025

Categorical data are presented as counts and percentages. Continuous data from normally distributed parameters are presented a as mean ± standard deviation, such as age, while continuous data not normally distributed are expressed as median (25% IQR, 75% IQR). LMWH, low-molecular-weight heparin; PNS, *Panax notoginseng* saponins; PT, prothrombin time; TT, thrombin time; APTT, activated partial thromboplastin time; INR, international normalized ratio; DVT, deep vein thrombosis; IQR, interquartile range.

expression, which all helps prevent the occurrence of thrombosis (48,50,51). Inhibition of PI3K/AKT signaling reduced platelet aggregation and thrombosis, while activation of PI3K/AKT signaling induced endothelial damage, apoptosis

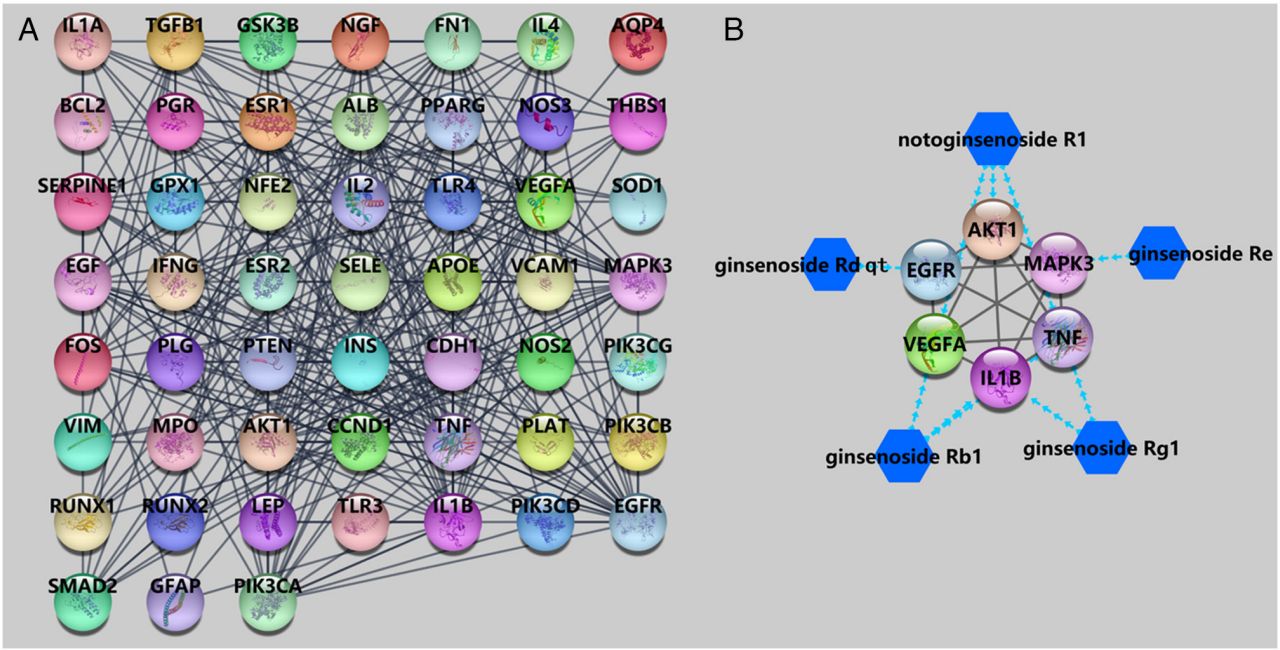

Figure 3. (A) Protein-protein interaction network and (B) main active compounds-hub target genes network.

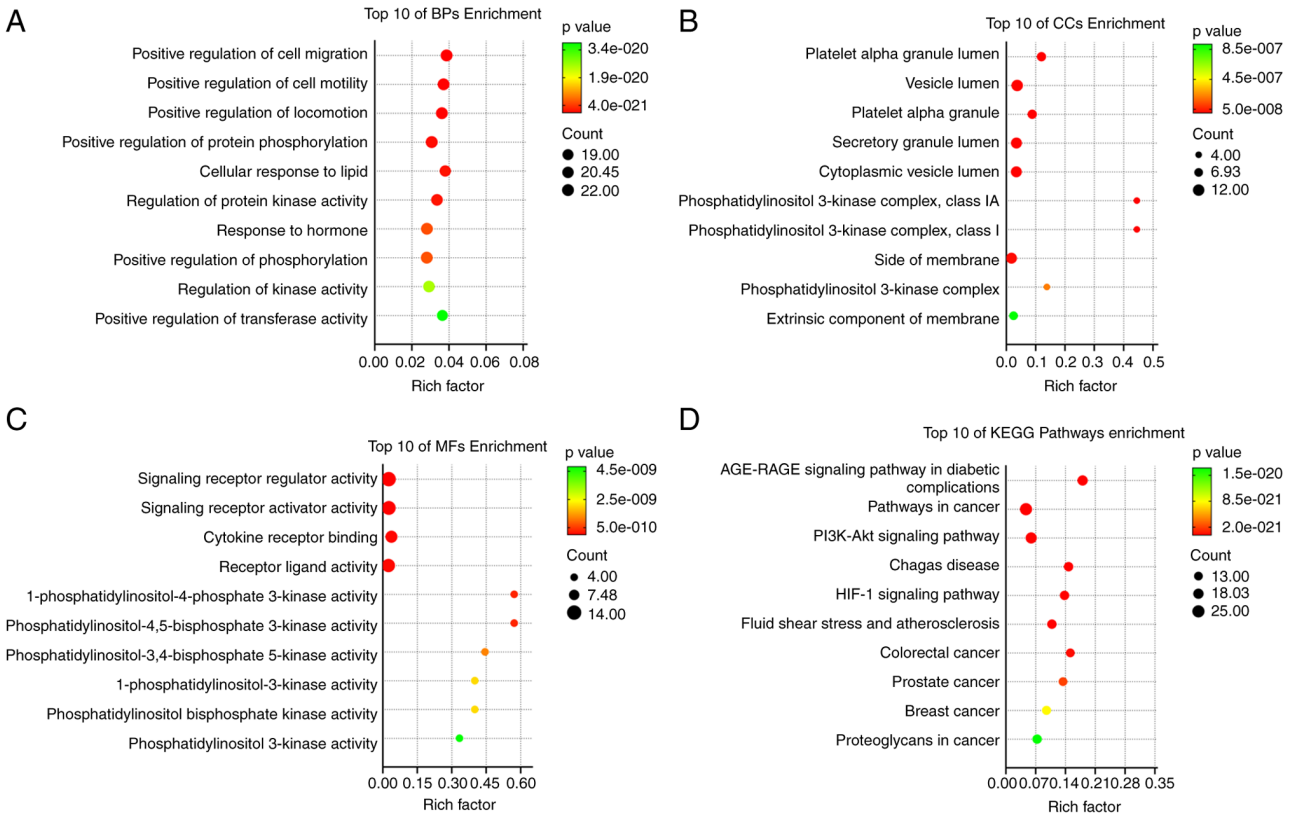

Figure 4. Top 10 most enriched Gene Ontology terms in the categories (A) BP, (B) CC and (C) MF and (D) Kyoto Encyclopedia of Genes and Genomes pathways. BP, biological process; CC, cellular component; MF, molecular function; HIF, hypoxia-inducible factor.

and inflammation (52-54). Integrins play a critical role in different phases of platelet function during thrombosis, being involved in both platelet-matrix interaction and platelet-platelet aggregation. The PI3K/Akt pathway regulates both integrin inside-out and outside-in signaling (55).

The five main active components of PNS are notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1 and ginsenoside Rd qt. Network pharmacology analysis identified the hub targets of these five main components in DVT prevention, and the spontaneous binding among them was

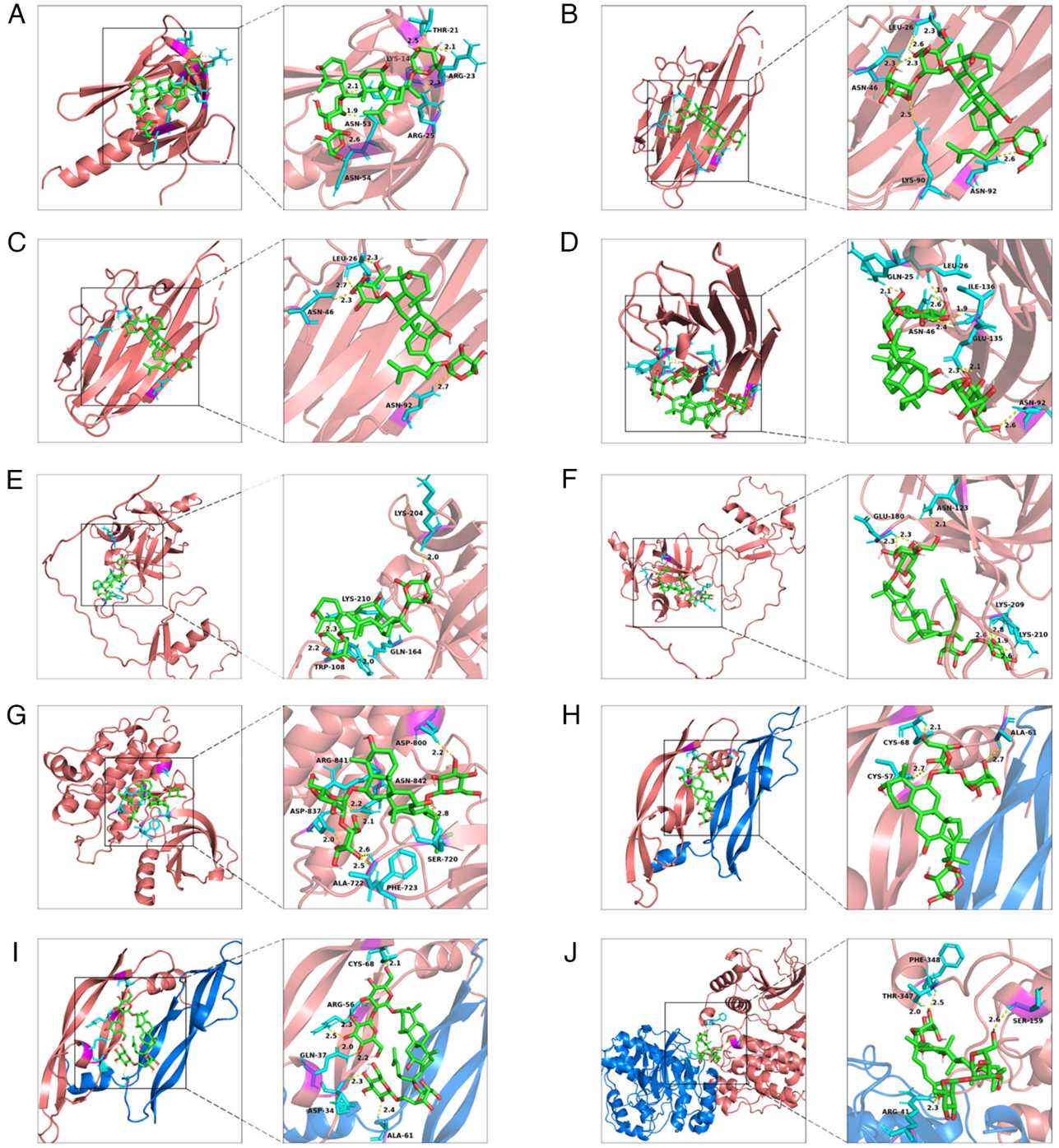

Figure 5. Molecular docking analysis. (A) AKT1 and notoginsenoside R1, (B) TNF and notoginsenoside R1, (C) TNF and ginsenoside Rg1, (D) TNF and ginsenoside Rb1, (E) IL1B and ginsenoside Rg1, (F) IL1B and ginsenoside Rb1, (G) EGFR and ginsenoside Rd qt, (H) VEGFA and notoginsenoside R1, (I) VEGFA and ginsenoside Rb1 and (J) MAPK3 and ginsenoside Re.

verified by molecular docking. A binding energy <0 kcal/mol indicates the docking molecule had spontaneous binding activity to the target, with a smaller value of binding energy reflecting a higher binding ability. All molecules showed a binding energy <-6 kcal/mol with the targets, indicating excellent spontaneous binding of the ligands to the receptors.

A limitation of the present study is that no experiments were performed to validate the network pharmacology results, particularly *in vivo* experiments. Although network pharmacology and clinical data have provided preliminary insights into the mechanisms and effects of PNS for DVT prevention, these results may not fully reflect the complex conditions within a living organism. The results of pure clinical data without *in vivo* experiments limit the clinical generalization of the present findings. Therefore, *in vivo* experiments may be performed in future studies by our group to more comprehensively evaluate the potential value of PNS in preventing DVT.

In conclusion, PNS can promote the effect of LMWH to prevent DVT. We identified potential targets and pathways

for PNS in the prevention of DVT, and a basis for subsequent experimental verification was provided. The hub targets of PNS in preventing DVT were AKT1, TNF, IL1B, EGFR, VEGFA and MAPK3. Molecular docking analysis showed that the main active components of PNS could combine well with these hub targets. The AGE-RAGE signaling pathway and the PI3K-Akt signaling pathway may be critical pathways for PNS to prevent DVT.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BY and YN conceptualized and supervised the study, validated and curated data and wrote, reviewed and edited the manuscript. JG developed the methodology, and performed software analysis and data validation, and reviewed and edited the manuscript. YN and JG checked and confirmed the authenticity of the raw data. LL conceptualized and supervised the study. CW performed formal analysis and project administration and validated the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Xuanwu Hospital, Capital Medical University (Beijing, China; approval no. [2017]088). All patients provided their verbal and written informed consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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