Efficacy of Renshen Sanqi Chuanxiong formula for preventing vascular aging

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Supported by the National Nature Science Foundation of China (the Mechanism research of Vascular Aging Induced by High Glucose from AMPK/mTOR Pathway and the Interfering Effects of Yiqi Huoxue Chinese Herbal Medicine, No. 81673822; the Molecular Mechanism Research of Yiqi Huoxue Fang Postponing Vascular Endothelial Cell Senescence by SIRT1-autophagy Pathway, No. 81503448); and the Independent Topic Program of China Academy of Chinese Medical Sciences (Effect of Intestinal Microflora on High Glucose-induced Vascular Aging and Intemention of Extracts from Radix Ginseng Radix Notoginseng and Rhizoma Chanxiong, No. ZZ2017011)

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Accepted: August 8, 2019

Abstract

OBJECTIVE: To evaluate the efficacy of Renshen Sanqi Chuanxiong formula (RSCF) for preventing vascular aging, and to investigate the possible molecular mechanism underlying the actions of RSCF.

METHODS: Potentially active components and their relatively direct targets were identified by combining drug-likeness (DL) screening using a target identification process. Vascular aging-associated targets for RSCF were obtained by selecting common genes not only from potential targets but also from human vascular aging-associated genes. Cytoscape 3.2.1 software was employed to visualize the complex compound-target and target-function networks. Biological process and molecular function were assessed, and the Kyoto Encyclopedias of Genes and Genomes and pathway enrichment analyses were performed using ClueGO. Pathways directly associated with vascular aging were integrated into a “vascular aging-related” pathway.

RESULTS: Altogether, 122 potentially active components of RSCF were identified through DL screening, and their corresponding 692 direct targets were retrieved via target prediction and identification. We identified 49 vascular aging-associated targets for RSCF by overlapping the 692 potential targets with 146 human vascular aging-associated genes. The results from the compound-target network indicated that most components acted on common targets and displayed synergistic action, which showed that the magnifying effects of RSCF were based on these common targets. The target-function network revealed that each target was involved in multiple function modules, suggesting that RSCF was multi-functional during treatment of vascular aging. The results of the ClueGO analysis indicated that most of the targets were associated with the hypoxia-inducible factor 1 (HIF-1) signaling pathway. The results from the pathway analysis also indicated that an integrative vascular aging-related pathway mainly included an angiogenesis
regulation module, cell-survival module, and oxidative stress-resistance module.

CONCLUSION: Our results suggested that many components act synergistically on common targets to delay vascular aging, and each target is involved in multiple functional modules. The ClueGO analysis indicated that most of the targets were connected to the HIF-1 signaling pathway, FOXO signaling pathway, and thyroid hormone signaling pathway.

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Keywords: Cardiovascular diseases; Pharmacology; Drug development; Vascular aging; Molecular mechanism; Renshen Sanqi Chuanxiong formula

INTRODUCTION

As “the world’s leading killer” of humans, cardiovascular diseases (CVDs) kill more than 10 million people worldwide every year, and the number of deaths is predicted to reach 23.6 million by 2030. The mortality rate attributed to CVDs increases with age, and vascular aging is recognized as a major risk factor for various CVDs. Vascular aging — characterized by reduced arterial compliance, increased stiffness, and impaired contraction — is due to degenerative changes in the vasculature. However, few western medicines have shown promising therapeutic effects on vascular aging.

Traditional Chinese Medicine (TCM) has been used for thousands of years in China under the guidance of a holistic therapeutic philosophy. TCM, with its multi-component and multi-target characteristics, is effective for synergistically and holistically treating complex diseases and is becoming increasingly popular worldwide. Even so, a lack of knowledge about TCM compared with modern medicine has kept it from being used in mainstream medicine.

Chinese herbal medicines that tonify Qi and activate blood circulation have been used successfully to delay vascular aging. One formula used for this purpose is Renshen Sanqi Chuanxiong formula (RSCF). RSCF is composed of Renshen (Radix Ginseng), Sanqi (Radix Notoginseng), and Chuanxiong (Rhizoma Chuanxiong). Our previous in vitro and in vivo studies showed that RSCF significantly prevented vascular aging. For example, RSCF effectively prevented vascular aging in naturally aged mice by inhibiting the oxidative stress pathway. In addition, RSCF dramatically regulated cytoskeleton protein during replicative senescence of vascular smooth muscle cells. Although RSCF was found to delay vascular aging efficiently, with inherent features of TCM (e.g., its multi-ingredient and multi-target characteristics) the molecular mechanism of RSCF remains unclear.

Systems biology coincides with the holistic view of TCM and is perceived to be an efficient tool for exploring the role of TCM. As part of the interdisciplinary fields of systems biology, pharmacology, and pharmacodynamics, systems pharmacology is based on analytical network processes and has been used successfully to evaluate the molecular mechanisms of some Chinese medicinal herbs and formulas. In the present study, we used a systems pharmacology approach that integrates chemical, pharmacokinetic, and pharmacological data to uncover the potential molecular mechanisms of RSCF in preventing vascular aging (Figure 1). This study aimed to evaluate the efficacy of RSCF for preventing vascular aging and investigate the possible molecular mechanism(s) underlying the actions.

MATERIALS AND METHODS

Chemical composition of RSCF

Structural information on the chemical ingredients of each herb in RSCF was obtained from the Traditional Chinese Medicine Systems Pharmacology Platform (TCMSP) (http://lbi.hkbu.edu.hk/lsp/tcmsp.php). TCMSP, a comprehensive systems pharmacology database for TCM, comprises 510 TCM herbs registered in the Chinese pharmacopoeia and > 33,000 compounds. The TCMSP includes herbal ingredients, predicted targets, associated drug-target-disease networks, and the predicted pharmacokinetic properties of the herbs’ ingredients.

Candidate compound screening

Drug likeness (DL), a qualitative concept, is used for drug design to evaluate the “drug-like” qualities of candidate compounds. Wang’s group developed a pre-DL model to calculate the DL values for each active ingredient by estimating the Tanimoto similarity between ingredients and those of clinical drugs in the Drugbank database (http://www.drugbank.ca/). To obtain compounds with favorable ADMET (absorption, distribution, metabolism, excretion, toxicity) properties, we further excluded compounds for which the DL value was < 0.18 because the average DL value of all molecules in the DrugBank is 0.18.

Target identification for RSCF

Identification of protein targets for candidate compounds in formulas plays a key role in systems pharmacology-based drug discovery. In this study, we performed target identification via known drug-target interactions (DTIs) mapping and predicted DTIs using balanced substructure-drug-target network-based inference (bSDTNBI). The known DTIs were identified by mapping candidate compounds in the formula onto a known DTIs network of natural products. This network contains 18,008 DTIs connecting 2988 unique natural products to 3546 targets.
In this study we calculated substructure items of each compound using four types of molecular fingerprints from PaDEL-Descriptor (version 2.18), including Substructure (FP4), Klekota-Roth (KR), MACCS, and PubChem (PubChem). L denotes to the top prediction lists (here, \( L = 50 \)). Four precision (P), recall (R), precision enhancement (\( e_P \)), and recall enhancement (\( e_R \)) were calculated as below.

\[
P(L) = \frac{1}{M} \sum_{i=1}^{M} \frac{X(L)}{L} \\
R(L) = \frac{1}{M} \sum_{i=1}^{M} \frac{X(L)}{X_i} \\
e_P(L) = P(L) \cdot \frac{M \cdot N}{X} \\
e_R(L) = R(L) \cdot \frac{N}{L}
\]

Where \( M \) and \( N \) represent the number of drugs and targets participated in performance evaluation. \( X(L) \) is the number of the correctly predicted DTIs which were ranked in the top \( L \) places of \( D_i \)’s newly predicted target list, \( X \) is the number of \( D_i \)’s DTIs that were divided into test set, \( X \) is the total number of DTIs that were divided into test set.

As an improved network-based method based on the original substructure-drug-target network-based inference,\(^{22}\) the bSDTNBI method utilizes resource-diffusion processes to prioritize potential targets for both known drugs and new chemical entities via a substructure-drug-target network.\(^{23}\) Among the four network models generated with different types of fingerprints, the bSDTNBI-KR model achieved the best performance with the highest values of \( P \) (0.049), \( R \) (0.752), \( e_P \) (27.02), \( e_R \) (27.24), and area under the curve (0.959).\(^{23}\) Thus, bSDTNBI KR was chosen as the predictive model to identify potential DTIs of natural products. The KR molecular fingerprint was calculated for each candidate compound. There were four parameters in the bSDTNBI KR. The first two parameters aimed to balance the allocation of the initial resource of different node types (\( \alpha \)) and the weighted values of different edge types (\( \beta \)), respectively. The third parameter \( \gamma \) was used to balance the influence of hub nodes in resource diffusion processes, and the fourth parameter \( k \) represented the number of resource diffusion processes. In this study, four parameters \((\alpha = \beta = 0.1; \ \gamma = -0.5; k = 2)\) using the top 50 (\( L \)) as a cutoff.

Figure 1 Flowchart based on a systematic pharmacological approach

A systematic pharmacology approach was constructed to identify and evaluate the active compounds and therapeutic mechanisms of Renshen Sanqi Chuanxiong formula (RSCF) to prevent vascular aging.

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**Figure 1** Flowchart based on a systematic pharmacological approach

A systematic pharmacology approach was constructed to identify and evaluate the active compounds and therapeutic mechanisms of Renshen Sanqi Chuanxiong formula (RSCF) to prevent vascular aging.
Manual curation of human vascular aging-associated genes

First, we collected human vascular aging-associated genes (VAAGs) from the National Center for Biotechnology Information (NCBI) gene database. The NCBI gene database (www.ncbi.nlm.nih.gov/gene) integrates gene-specific information from multiple data sources. Thereafter, we gathered AAGs from two comprehensive aging databases: the JenAge Ageing Factor Database (AgeFactDB, http://agefactdb.jenage.de) and the Human Ageing Genomic Resources (HAGR, http://genomics.senescence.info) database. AgeFactDB integrates aging phenotype data using both experimental and computational evidence, whereas the HAGR database collects AAGs from experimental evidence. In this study, we collected AAGs with well-defined experimental evidence across Homo sapiens species from the AgeFactDB and HAGR databases and then removed the duplicated AAGs between the databases.

Network construction

A local (known) compound-target (C-T) network, a global (known and predicted) C-T network, and a target-function network (T-F network) were constructed to interpret the multi-ingredient, multi-target, and multi-function characteristics of RSCF for treating vascular aging. In these networks, the nodes represented compounds/targets-functional modules, and the edges indicated how they are connected to each other. Cytoscape 3.2.1 software, an open source for visually integrating biomolecular interaction networks, was used to visualize the complex C-T and T-F networks.

Pathway construction and analysis

To investigate how the biological effects of targets affect disease by modulating specific pathways, an integrated vascular aging-related pathway was constructed based on the present knowledge of vascular aging pathology. Briefly, the predicted targets were first mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and then divided into several pathways. Thereafter, the pathways closely associated with vascular aging-related diseases were selected and incorporated into an integrated vascular aging-related pathway using the pathological and clinical results.

RESULTS

Filtering of candidate compounds

In this study, the 498 chemical ingredients found in RSCF were derived from the TCMSP, including RS (190), SQ (119), and CX (189). After excluding the compounds with DL values < 0.18, we selected 122 unique candidate compounds with appropriate pharmaceutical properties as the potentially active compounds. The numbers of active compounds in RS, SQ, and CX were 86, 27, and 20, respectively.

Target identification and manual curation of human VAAGs for RSCF

After known drug-target mapping and drug-target prediction using bSDTNBI, 692 potential targets were identified in the 122 candidate compounds. In addition, we pooled 1726 human vascular-associated genes from the NCBI gene database and 309 human AAGs from the AgeFactDB and HAGR databases, and then generated 146 VAAGs for our systems pharmacology-based analysis (Figure 2). Finally, we identified 49 VAAGs for RSCF by overlapping the 692 potential targets with the 146 human VAAGs.

Figure 2 Overlap among 1726 human vascular-associated genes from the NCBI gene database and 309 human AAGs from the AgeFactDB and HAGR databases.

AAGs: aging-associated genes; AgeFactDB: JenAge Ageing Factor Database; HAGR: Human Ageing Genomic Resources; NCBI: National Center for Biotechnology Information.

Analysis of synergistic action of candidate targets of RSCF

To analyze the synergistic action of the candidate targets of RSCF, we evaluated the overlap of the vascular aging-associated targets for the three distinct herbs (42 from RS, 30 from SQ, and 29 from CX), using IntegrativeVenn (http://www.interactivenn.net/).

As shown in Figure 3, the targets of the three herbs included 49 vascular aging-associated targets in total. In addition, 21 were common targets for all three herbs, which showed that RSCF performed its magnifying effects based on common targets. Furthermore, we found that any two of the three herbs displayed synergistic action based on the common targets. For example, RS and SQ shared 26 common vascular aging-associated targets.

C-T network

Figures 4 and 5 show the interactions between compounds and their corresponding vascular aging-associated targets. As shown in Figure 4, there are 167 known C-T interactions connecting 113 targets. Remarkably, 23 (20.35%) were associated with vascular aging, indicating that regulation of vascular aging is a major effect of RSCF. Figure 5 presents a global (known and predicted) C-T network, consisting of 756 C-T interactions linking 121 candidate compounds to 49 vascular aging-associated targets. This network indi-
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Figure 3 Overlap of vascular aging-associated targets among three herbs in RSCF

The corresponding numbers of targets for each herb were 40 (Renshen), 30 (Sanqi), and 29 (Chuanxiong). RSCF: Renshen Sanqi Chuanxiong formula.

...indicated that the average target degree (D) value (the number of candidate compounds related to it) was 6.2 and the mean drug degree (K) value (the number of targets related to it) was 15.4. Table 1 shows the top 50 ranked potential compounds in RSCF according to target degree. Among these compounds, M001 (querceatin) had the highest number of targets (D = 18), followed by M023 (senkyunone, D = 11), M006 (kaempferol, D = 10), and M016 (levistolid A, D = 10). These compounds together showed the multi-target properties of substances involved in the activity of RSCF. Table 2 shows the data on vascular aging-related targets of RSCF and their degree parameters in C-T networks. Among these target proteins, the androgen receptor (AR, K = 111) was targeted by the largest number of compounds, followed by nuclear factor erythroid 2-related factor 2 (NFE2L2, K = 87), tyrosine-protein phosphatase non-receptor type 1 (K = 78), hypoxia-inducible factor 1α (HIF1A, K = 61), and cannabinoid receptor 1 (cannabinoid receptor 1, K = 56). These results indicate that the multi-component properties of TCM are involved in RSCF activity.

T-F network

As shown in Figure 6, the T-F network consists of 49 candidate targets, 8 vascular aging-related functional modules, and the corresponding 269 interactions. More specifically, vascular aging-related functional...
modules mainly include blood circulation and the regulation of cell aging, enzyme activities, cytothesis, cell death, metabolic activities, and immune/inflammation responses. T-F network analysis suggested that each target was involved in an average of 5.49 functional modules, and 15 of the 49 targets were related to more than 5 functional modules.

**ClueGO analysis of vascular aging-associated targets**

As shown in Figure 7, the biological process mainly consists of four types of processes: cellular response to oxygen-containing compounds; negative regulation of apoptotic processes; regulation of monooxygenase activity; positive regulation of DNA metabolic processes. Thus, most vascular aging-related targets are associated with redox reactions and apoptotic-relevant processes (Figure 7A and B). The molecular functions of the targets fall into four types: transferase activity (transferring phosphorus-containing groups); transcription factor binding; organic cyclic compound binding; ubiquitin protein ligase binding (Figure 7C and D). Finally, KEGG pathway enrichment analysis indicated that most of the targets are associated with the hypoxia-inducible factor 1 (HIF-1) signaling pathway, thyroid hormone signal pathway, and Forkhead box protein O (FOXO) signaling pathway (Figure 7E and F). Furthermore, previous data suggested that the HIF-1 signaling pathway played a critical protective role in the pathophysiology of cardiovascular diseases by regulating angiogenesis, vascular remodeling, and oxygen utilization.

**Pathway analysis**

As shown in Figure 8, this vascular aging-associated pathway can be classified into several functional models, including angiogenesis, cell proliferation, cell survival, oxidative stress resistance, and DNA repair.

**DISCUSSION**

Among the 21 common targets for all three herbs, cholesteryl ester transfer protein plays a role in the regulation of high-density lipoprotein metabolism. Previous data indicated that inhibition of cholesteryl ester transfer protein effectively increased high-density lipoprotein concentrations, which decreased the incidence and progression of coronary artery disease. Simultaneously, preclinical and clinical studies indicated that suppression of cannabinoid receptor 1 improved glucose and lipid homeostasis, thereby contributing to an increase in cardiovascular risk. In addition, transient
Table 1 Top 50 ranked potential compounds in Renshen Sanqi Chuanxiong formula according to target degree

<table>
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<th>Molecule-ID</th>
<th>Compound</th>
<th>Inchikey</th>
<th>Degree</th>
<th>Source</th>
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Notes: SQ: Sanqi; CX: Chuanxiong; RS: Renshen.
Table 2 Vascular aging-related targets of Renshen Sanqi Chuanxiong formula and their degree parameters in a compound-target network

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<th>UniProt ID</th>
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<th>Target name</th>
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<td>CNR1</td>
<td>Cannabinoid receptor 1</td>
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<td>PPARG</td>
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<td>ESRI</td>
<td>Estrogen receptor</td>
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<td>PRKCA</td>
<td>Protein kinase C alpha type</td>
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<td>Prostaglandin G/H synthase 2</td>
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<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
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<td>TP53</td>
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<td>Plasminogen activator inhibitor 1</td>
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<td>P60484</td>
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<td>IL2</td>
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<td>ATM</td>
<td>Serine-protein kinase ATM</td>
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Note: the organism in each case was human.
receptor potential cation channel subfamily V member 1 (TRPV1) is a potential therapeutic target for treating an array of cardiovascular diseases by improving vascular function. Experimental data indicated that activation of TRPV1 led to an increase in intracellular calcium and activation of protein kinase A and epithelial nitric oxide synthase, which led to vasodilation. These results showed that these common targets identified for RSCF coordinated to decrease cardiometabolic risk and enhance vascular function during treatment of vascular aging-related diseases.

According to the results of C-T network, the predicted compound-target interactions may provide meaningful guidance for treating vascular aging and its associated diseases. For instance, NFE2L2 is involved in vasoprotection by detoxifying reactive oxygen species and removing damaged proteins. Previous studies indicated that the activation of NFE2L2 by an NFE2L2 activator, such as sulforaphane, had great therapeutic potential for addressing vascular disease and aging, indicating that the regulation of NFE2L2 by active natural products, such as M001 (quercetin) and M003 (sito-glucoside), would be beneficial for preventing vascular aging. Additionally, experimental evidence showed that testosterone targeted ARs and regulated vascular endothelial function and the synthesis and bioavailability of nitric oxide. Interestingly, the morbidity from age-related diseases increases with the aging-associated decline in testosterone levels, indicating that AR activity, intensified by active compounds such as M006 (kaempferol) and M023 (senkyunone), may have a therapeutic effect on cardiovascular diseases. Furthermore, HIF-1α is considered a master regulator of hypoxic/ischemic vascular responses, boosting transcriptional activation of the hundreds of genes involved in vascular reactivity, angiogenesis, arteriogenesis, and the mobilization and homing of bone marrow-derived angiogenic cells. Previous preclinical studies indicated that increased HIF-1α activity restored normal physiological responses after hypoxia. Therefore, the regulatory effect of M010 (furmarine) and M019 (augustic acid) on HIF-1α may restore vascular homeostasis and contribute to the treatment of vascular diseases.

Figure 6 indicates that RSCF modulates an array of enzyme activities, including both oxidoreductase and monooxygenase activity. Emerging evidence has indicated that pathological oxidative stress associated with endothelial dysfunction is a major determinant of vascular aging. Therefore, regulation of oxidoreductase activity could be an effective method for treating vascular aging-related diseases.

In this study, we focused on three representative modules to explore the underlying therapeutic effects of the formula.

**Angiogenesis regulation module**
Arterial aging has been reported to impair vascular en-
Figure 7 ClueGO analysis of the vascular aging-associated targets
A functionally grouped network of enriched categories was built based on the target genes and the partially overlapped, functionally associated groups. GO terms are expressed as nodes, and the node size represents the term enrichment significance. The node pie charts represent molecular function, biological processes, cell location, and pathway analysis. Only the most significant terms in the groups are marked. A, B: representative biological process interactions among target genes. C, D: representative molecular function interactions among target genes. E, F: representative Kyoto Encyclopedia of Genes and Genomes (KEGG) database pathway analysis among target genes.
dothelial function and angiogenesis, which contributes to the increasing morbidity and mortality from cardiovascular diseases. Therefore, targeting angiogenesis is considered a promising therapeutic approach for many ischemia-induced cardiovascular diseases. Angiogenesis can effectively repair damaged tissue by increasing the blood supply to the heart and restoring energy to normal. As shown in Figure 8, target proteins involved in the HIF-1 signaling pathway that are modulated by herbal ingredients affected angiogenesis. M044 and M085 were both predicted to interact with HIF-1α and the signal transducer and activator of transcription 3 (STAT3). Previous studies indicated that the adenoviral HIF-1α gene transfers induced beneficial angiogenesis in vivo by increasing capillary size and energy recovery, whereas reduced HIF-1α expression decreases angiogenesis. Animal model evidence showed that activation of STAT3 necessarily promoted angiogenesis, which suggests an important role for STAT3 in blood vessel remodeling. Altogether, these studies indicated that the multitudinous treatment interventions of herbal formulas for vascular aging-associated diseases occurs by regulation of angiogenesis.

**Cell survival module**

Increased apoptotic cell death is thought to contribute to that age-related microvascular rarefaction that has been observed in multiple organ systems, leading to coronary resistance and reserve dysfunction. Hence, regulation of apoptosis is an effective approach to vascular protection. As seen in Figure 8, target genes regulated by active compounds through the PI3K-Akt signaling pathway are involved in cell survival modulation. Furthermore, M007, M016, and M017 regulates cellular tumor antigen p53 (p53), and previous studies have indicated that suppression of p53 attenuates apoptosis. Moreover, NF-κB plays a central role in promoting cell survival during the human endothelial cell response to serum withdrawal and tumor necrosis factor cytotoxicity. Therefore, modulation of NF-κB by M010 and M022 may result in an anti-apoptosis effect.

**Oxidative stress resistance and DNA repair module**

Oxidative stress and DNA damage contribute to cardiovascular disease by driving cellular senescence and cause a host of autocrine and paracrine alterations that contribute to cardiovascular disease. Thus, therapeutic efforts to address vascular aging should focus on targeting oxidative stress and DNA repair. As shown in Figure 8, target proteins labeled in the FOXO signaling pathway were linked to oxidative stress resistance and DNA repair. Previous preclinical studies indicated that activation of FOXO was modulated by reactive oxygen species-activated kinases, such as JNK. JNK serves as an upstream regulator of FOXO, which could lead to nuclear translocation and resistance to oxidative stress-induced cellular apoptosis. Therefore, activation of JNK by M065 may modulate oxidative stress and DNA repair-relevant cellular senescence. In addition, it has been reported that loss of FOXO or ataxia telangiectasia mutated (ATM) kinase has led to DNA damage and increased reactive oxygen species in cells, indicating that the increase in ATM activity regulated by M053 and M121 may modulate oxidative stress and DNA repair-relevant vascular disease.

In conclusion, the C-T network in this study indicated that many components acted synergistically on common targets to delay vascular aging, and the T-F network showed that each target was involved in multiple functional modules. Furthermore, the ClueGO analysis indicated that most of the targets were connected to...
the HIF-1 signaling pathway, FOXO signaling pathway, and thyroid hormone signaling pathway. Finally, pathway analysis revealed that angiogenesis regulation, cell survival, oxidative stress resistance, and DNA repair modules are representative modules for further interpretation of how RSCF synthetically regulates the vascular aging-related pathway.

However, there were some limitations in this study. First, we selected a DL value of $\geq 0.18$ as the filter criterion for candidate compounds, which may have incurred a risk of error or bias in the screening results. In fact, compounds with low DL values ($< 0.18$) were assumed less likely to be drugs. However, a few compounds with DL scores $< 0.18$ displayed certain biological activity. Therefore, it is necessary to further define the filter criterion for compounds in the future. Second, most of the targets for RSCF were derived by target prediction using the innovative bSDTNBI method, which lacks experimental validation. Hence, we plan to carry out experiments to verify the reliability and accuracy of this method for predicting targets.

Overall, the systematic pharmacological approach constructed in this work may provide a new method for scientifically evaluating the holistic and synergic essence of TCM for curing and preventing complex diseases.

REFERENCES