A network pharmacology approach to investigate the pharmacological effects of Guizhi Fuling Wan on uterine fibroids

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Abstract. To investigate the pharmacological mechanism of Guizhi Fuling Wan (GFW) in the treatment of uterine fibroids, a network pharmacology approach was used. Information on GFW compounds was collected from traditional Chinese medicine (TCM) databases, and input into PharmMapper to identify the compound targets. Genes associated with uterine fibroids genes were then obtained from the GeneCards and Online Mendelian Inheritance in Man databases. The interaction data of the targets and other human proteins was also collected from the STRING and IntAct databases. The target data were input into the Database for Annotation, Visualization and Integrated Discovery for gene ontology (GO) and pathway enrichment analyses. Networks of the above information were constructed and analyzed using Cytoscape. The following networks were compiled: A compound-compound target network of GFW; a herb-compound target-uterine fibroids target network of GWF; and a compound target-uterine fibroids target-other human proteins protein-protein interaction network, which were subjected to GO and pathway enrichment analyses. According to this approach, a number of novel signaling pathways and biological processes underlying the effects of GFW on uterine fibroids were identified, including the negative regulation of smooth muscle cell proliferation, apoptosis, and the Ras, wingless-type, epidermal growth factor and insulin-like growth factor-1 signaling pathways. This network pharmacology approach may aid the systematical study of herbal formulae and make TCM drug discovery more predictable.

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Abbreviations: GFW, Guizhi Fuling Wan; GO, gene ontology; TGF-β, transforming growth factor-β; Wnt, wingless-type; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; UAE, uterine artery embolization; MRgFUS, magnetic resonance imaging-guided focused ultrasound surgery; NSAIDs, non-steroidal anti-inflammatory drugs; GnRHa, gonadotropin releasing hormone agonists; TCM, traditional Chinese medicine; PPI, protein-protein interaction; IGF, insulin-like growth factor; PCNA, proliferating cell nuclear antigen; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; RTKs, receptor tyrosine kinase; RA, retinoic acid; SPRM, selective progesterone receptor modulator; PI3K, phosphoinositide 3-kinase; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor-κB; Erk, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; ER, estrogen receptor; PR, progesterone receptor; Bcl-2; B-cell lymphoma 2

Key words: Guizhi Fuling Wan, uterine fibroids, mechanism, formula, network pharmacology

Introduction

Uterine fibroids is the most common type of tumor to occur in the female reproductive tract, and develops from the clonal proliferation of single smooth muscle cells of the myometrium, initially caused by cellular genetic changes (1,2). Alterations in complex signaling pathways involving factors such as steroids, growth factors, transforming growth factor-β (TGF-β)/Smad, wingless-type (Wnt)/β-catenin, retinoic acid (RA) serve an important role in the development of uterine fibroids (3,4). Furthermore, these signaling factors may regulate multiple pathways through common factors, such as mitogen-activated protein kinase (MAPK) and protein kinase B (also known as Akt) (4). The major risk factors that promote tumor development include genetic, hormonal, immunological and environmental factors (1-4). At present, besides surgical approaches such as hysterectomy, myomectomy, uterine artery embolization (UAE) and magnetic resonance imaging-guided focused ultrasound surgery (MRgFUS) (5), the pharmacological strategies for uterine fibroids focus on relieving symptoms and slowing or arresting tumor development. Three main types of therapeutic are used to reduce symptoms and inhibit the proliferation of fibroids (6,7), namely non-steroidal anti-inflammatory drugs (NSAIDs) (8), gonadotropin releasing hormone agonists (GnRHa) (9) and synthetic steroids with antiprogesterone activity, including mifepristone and asoprisnil (10). As a therapeutic strategy, surgery is associated with operative mortality and morbidity (11), and medicinal therapy is similarly limited because of its side effects. GnRHa

may relieve bleeding and bulk-related symptoms, but may also cause significant menopausal side effects (12,13). Furthermore, progesterone antagonists and other hormonal therapies that alter estrogen and progesterone production or function may affect fertility (14).

As an important component of complementary and alternative medicine, Chinese herbal formulas, including Guizhi Fuling Wan (GFW), are utilized for the treatment of uterine fibroids (15). A previous bibliometrics study of modern literature that analyzed the names of diseases treated with Guizhi Fuling pills demonstrated that the formula was most frequently used to treat abdominal masses (zheng jia) in traditional Chinese medicine (TCM) and uterine fibroids in western medicine (15).

GFW was first described in Essential Prescriptions from the Golden Cabinet (Jingui Yaolue) (16). The traditional effects of the GFW formula are considered to be invigoration of the blood, prevention of blood stasis and reduction of masses. GFW is composed of *Cinnamomi Ramulus* (*Gui Zhi*), *Poria Cocos (Schw.) Wolf.* (*Fuling*), *Cortex Moutan* (*Mu Dan Pi*), *Radix Paeoniae Rubra* (*Chi Shao*) and *Persicae Semen* (*Tao Ren*) (16,17). A previous systematic review of 38 randomized controlled trials involving 3,816 participants demonstrated that compared with mifepristone alone, GFW or GFW plus mifepristone reduced the volume of fibroids and improved dysmenorrhea to a greater extent, and were considered to be safer (18). This suggests that GFW may be a potential alternative medicine for the treatment of uterine fibroids; however, its pharmacological mechanism is not well understood.

Chinese formulas are multi-target, multi-component recipes that achieve their specific therapeutic efficacy through active components that regulate molecular networks within the body (19). Therefore, novel methods and tactics are required to systematically investigate and explain the mechanism of Chinese formulas. Network pharmacology, combined with pharmacology and pharmacodynamics, is a novel research field that is involved in the application of omics and systems biology-based technologies (20). As Chinese formulas are considered to have multiple targets, pathways, components and specificities, as necessary factors for the treatment of multiple complex illnesses, network pharmacology methods are suitable for investigating prior knowledge regarding the combination rules of TCM herbal formulae (21). Based on a previous study by Zheng *et al* (22), the present study selected a network pharmacology approach to uncover the pharmacological mechanism of GFW in the treatment of uterine fibroids.

Materials and methods

Data preparation

Composite compounds of the GFW herbs. To collect data on the compounds of GFW, the TCM Database@Taiwan (http://tcm.cmu.edu.tw/zh-tw/), as a comprehensive TCM database previously used to identify TCMs for osteoarthritis (23), and the Traditional Chinese Medicine Systems Pharmacology Database (ibts.hkbu.edu.hk/LSP/tcmsp.php), as a systems pharmacology platform for Chinese herbal medicines (24), were used, along with related literature (25). A total of 565 herbal compounds were identified; 230 in *Cinnamomi Ramulus*, 55 in *Poria Cocos (Schw.) Wolf.*, 78 in *Cortex Moutan*, 135 in *Radix Paeoniae Rubra* and 67 in *Persicae Semen*. Combined with related literature (25), 28 active compounds were identified: Cinnamic acid, cinnamic aldehyde, 3-(2-methoxyphenyl)-2-propenal, polyporenic acid C, pachymic acid, dehydrotrametenolic acid, trametenolic acid, gallic acid, oxypaeoniflorin, (+)-catechin, apiopaeonoside, paeonilide, paeoniflorin, suffruticoside B, suffruticoside D, galloylpaeoniflorin, tetragalloylglucopyranose, pentagalloylglucopyranose, benzoic acid, mudanpioside H, hexagalloylglucopyranose, benzoyloxypaeoniflorin, mudanpioside C, benzoylalbiflorin, paeonol, benzoylpaeoniflorin, albiflorin and amygdalin.

Compound target of each GFW herb. All the active compounds were input into SciFinder (http://scifinder.cas.org), a database of chemical and bibliographic information provided by the Chemical Abstracts Service (Columbus, OH, USA), and the molecular structure of each compound was obtained. The structures were drawn in ChemBioDraw 14.0 (PerkinElmer, Inc., Waltham, MA, USA) and saved as 'mol2' file format. These files were imported into PharmMapper (lilab.ecust.edu.cn/pharmmapper), a web server that uses a pharmacophore mapping approach for potential drug target identification (26). Using this web server, the targets of all compounds were identified, excluding that of cinnamic aldehyde as the target data for cinnamic aldehyde was not obtained via the PharmMapper prediction. Due to non-standard naming of the compound targets, the UniProt Knowledgebase (www.uniprot.org/) was used. The protein names were input with the species limited to 'homo sapiens' to obtain official symbols. Following these procedures, the compound targets with official symbols were obtained from the UniProt Knowledgebase.

Uterine fibroids targets. Genes associated with uterine fibroids were identified with two resources; i) GeneCards (www.genecards.org), as a database containing information on genes and their products and biomedical applications, provided by the Weizmann Institute of Science (Rehovot, Israel) and ii) the Online Mendelian Inheritance in Man database (www.omim.org), which catalogues all known diseases with a genetic component and, when possible, links them to relevant genes in the human genome, providing references for further research and tools for genomic analysis of catalogued genes (27). These databases were searched using the keywords 'uterine fibroids', 'uterine fibromas' and 'leiomyoma uterine', which identified a total of 114 genes.

Protein-protein interaction (PPI) data. Data on PPIs was obtained from STRING (http://string-db.org/; version 10), with the species limited to 'homo sapiens' and a confidence score >0.4, and InAct (www.ebi.ac.uk/intact/; version 4.2.4). STRING is a database of known and predicted protein-protein interactions (28) and InAct provides an open source database and analytical tool for molecular interaction data (29).

Network construction

Network construction method. The following networks were constructed: A compound-compound target network of GFW; a herb-compound target-uterine fibroids target network of GFW; and a compound target-uterine fibroids target-other human proteins PPI network. The network analysis software Cytoscape (www.cytoscape.org; version 3.2.1) was used to

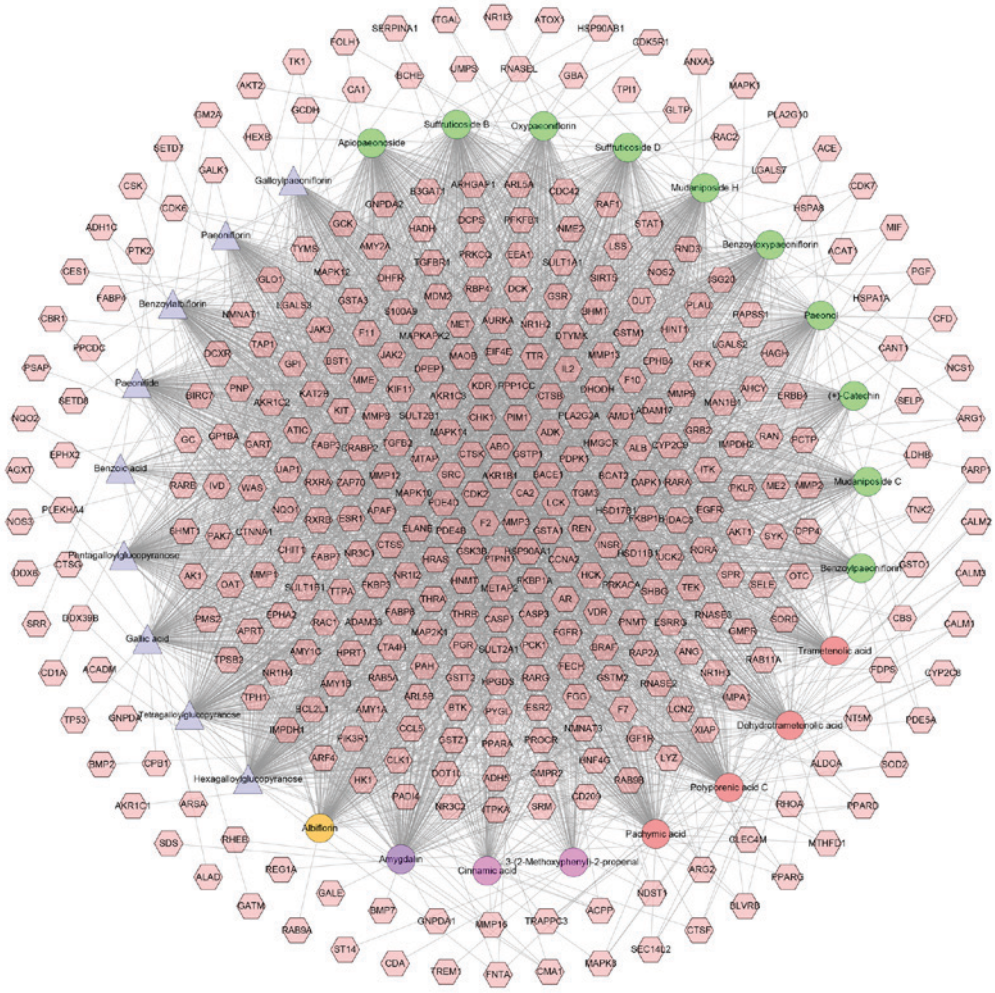

Figure 1. Compound-compound target network of Guizhi Fuling Wan. This network comprised of 362 compound targets and 27 compounds. Pink hexagons represent the compound targets; pink, red, green, orange and purple circles indicate compounds of *Cinnamomi Ramulus*, *Poria Cocos (Schw.) Wolf.*, *Cortex Moutan*, *Radix Paeoniae Rubra* and *Persicae Semen*, respectively; and blue triangles indicate the common compounds of *Cortex Moutan* and *Radix Paeoniae Rubra*.

construct networks. Cytoscape is a software that may be used to visualize biological pathways and intermolecular interaction networks, among others. Furthermore, it provides a basic set of features for data integration, analysis and visualization for complicated network analysis (30).

Network topological feature set definition. The nodes in each network were evaluated based on three indices: Degree, node betweenness and node closeness. Degree indicates the number of edges between a node and other nodes in a network (31). Node betweenness evaluates the participation of a node in the shortest parts of a network and reflects the capability of nodes to manage the rate of information flow in the network (32). Node closeness represents the inverse of the sum of the distance from node i (any given node) to other nodes (33). The importance of a node in a network is indicated by the values of these indices, with higher values indicating greater importance (22).

Enrichment analysis. Gene ontology (GO) and pathway enrichment analyses were also performed on the target data, using the Database for Annotation, Visualization and Integrated Discovery (DAVID; david.ncifcrf.gov/; version 6.8) (34). P-values were derived from the DAVID database and are modified Fisher exact P-values. Smaller P-values indicated greater enrichment (34).

Results

Compound-compound target network analysis. All active compounds, their targets and the interactions between them (excluding cinnamic aldehyde) are presented in Fig. 1. This network includes 389 nodes (362 compound targets and 27 compounds) and 3,500 edges. Nodes closer to the center exhibit more interactions with compounds than peripheral nodes, which indicates that numerous compound targets may be regulated by multiple compounds rather than a single compound. Coagulation factor II (also known as prothrombin), matrix metalloproteinase 3, carbonic anhydrase 2, aldo-keto reductase family 1 member B and cyclin dependent kinase 2 (in Fig. 1, F2, MMP3, CA2, AKR1B1 and CDK2, respectively) may be controlled by all of the active compounds.

Herb-compound target-uterine fibroids target network analysis. To understand the relationship between herbs of the GFW formula, compound targets and uterine fibroids targets, a herb-compound target-uterine fibroids target

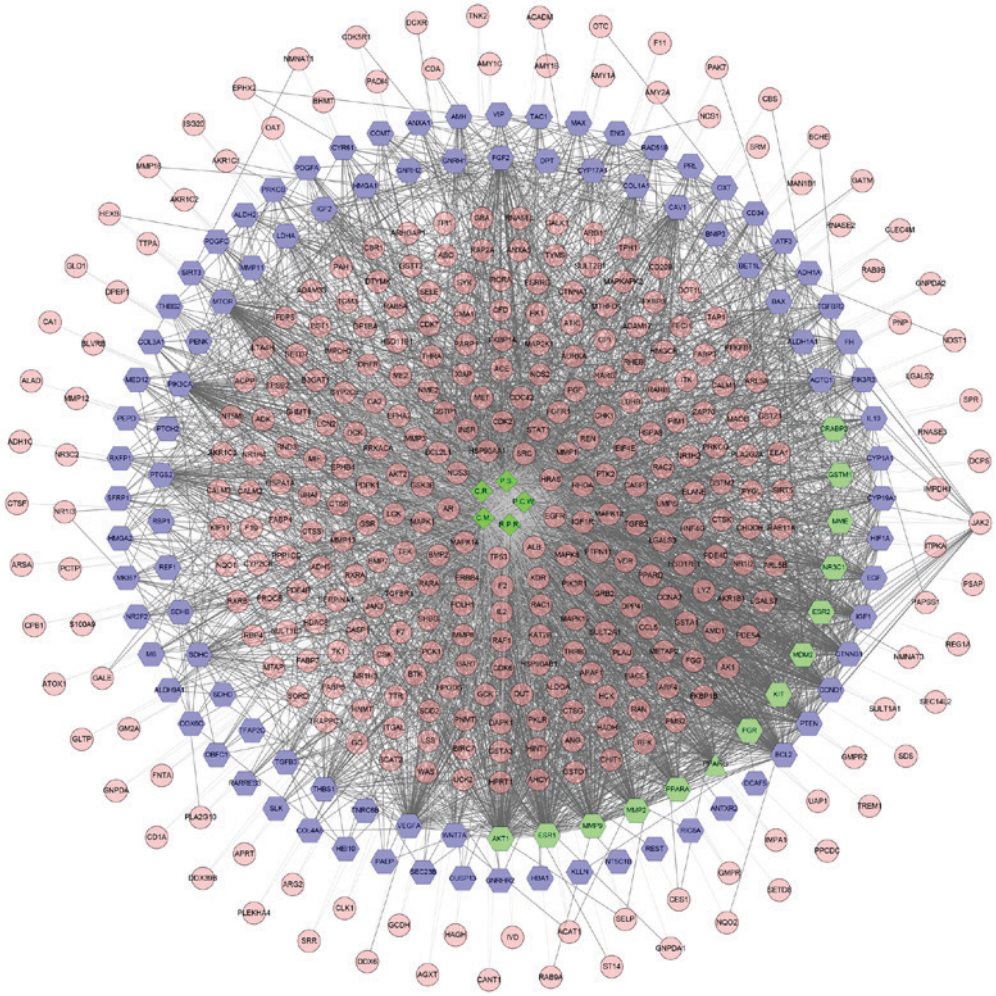

Figure 2. Herb-compound target-uterine fibroids target network of Guizhi Fuling Wan. Green diamonds, pink circles, blue hexagons and green hexagon represent the herbs, compound targets, uterine fibroids targets and compound-uterine fibroids targets, respectively; light lines indicate associations between herbs and other nodes; and dark lines indicate associations between fibroids targets, compound-uterine fibroids targets and compound targets. C.R., *Cinnamomi Ramulus*; C.M., *Cortex Moutan*; P.S., *Persicae Semen*; P.C.W., *Poria Cocos (Schw.) Wolf.*; R.P.R., *Radix Paeoniae Rubra*.

network was constructed. It was composed of 459 nodes (5 herbs, 362 compound targets, 78 uterine fibroids targets and 14 compound-uterine fibroids targets) and 3,736 edges (Fig. 2).

According to GO enrichment analysis, these targets were significantly associated with the steroid hormone-mediated signaling pathway (GO ID: 0043401; fold enrichment=16.1; P<0.001), response to estrogen (GO ID: 0043627; fold enrichment=8.4; P<0.001), response to estradiol (GO ID: 0032355; fold enrichment=7.5; P<0.001), response to progesterone (GO ID: 0032570; fold enrichment=6.4; P<0.001), vascular endothelial growth factor (VEGF) receptor signaling pathway (GO ID: 0048010; fold enrichment=5.3; P<0.001), epidermal growth factor (EGF) receptor signaling pathway (GO ID: 0007173; fold enrichment=4.3; P<0.001), fibroblast growth factor (FGF) receptor signaling pathway (GO ID: 0008543; fold enrichment=4.4; P<0.001), insulin-like growth factor (IGF) receptor signaling pathway (GO ID: 0048009; fold enrichment=10.4; P=0.0058), negative regulation of cell proliferation (GO ID: 0008285; fold enrichment=3.1; P<0.001), negative regulation of smooth muscle cell proliferation (GO ID: 0048662; fold enrichment=8.9; P<0.001), RA receptor signaling pathway (GO ID: 0048384; fold enrichment=15.1; P<0.001), response to vitamin A (GO ID: 0033189; fold enrichment=13.5; P<0.001), response to RA (GO ID: 0032526; fold enrichment=6.2; P<0.001), TGF-β receptor signaling pathway (GO ID: 0007179; fold enrichment=4.1; P<0.001), negative regulation of the TGF-β receptor signaling pathway (GO ID: 0030512; fold enrichment=4.4; P<0.001), positive regulation of apoptotic processes (GO ID: 0043065; fold enrichment=2.8; P<0.001) and apoptotic processes (GO ID: 0006915; fold enrichment=2.0; P<0.001; Table I and Fig. 3).

Through pathway enrichment, it was observed that compound targets and uterine fibroids targets were primarily related to the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway (fold enrichment=3.0; P<0.001), Ras signaling pathway (fold enrichment=3.6; P<0.001), MAPK signaling pathway (fold enrichment=2.3; P<0.001), estrogen signaling pathway (fold enrichment=4.7; P<0.001), VEGF signaling pathway (fold enrichment=6.7; P<0.001), mechanistic target of rapamycin (mTOR) signaling pathway (fold enrichment=5.1; P<0.001), Wnt signaling pathway (fold enrichment=1.9; P=0.032), apoptosis (fold enrichment=3.6; P<0.001), EGF signaling pathway (fold enrichment=3.8; P<0.001), IGF-1 signaling pathway (fold enrichment=4.5; P<0.001), role of ErbB2 receptor tyrosine

Table I. GO enrichment analysis of compound targets, uterine fibroids targets and compound targets/uterine fibroids targets.

GO ID	Pathway	Gene count	%	P-value	Fold enrichment	Benjamini
0043401	Steroid hormone mediated signaling	26	0.040	4.80E-24	16.1	5.60E-21
0032355	Response to estradiol	24	0.037	9.40E-14	7.5	2.50E-11
0043627	Response to estrogen	15	0.023	1.70E-9	8.4	1.40E-7
0032570	Response to progesterone	7	0.011	6.90E-4	6.4	1.30E-2
0048010	Vascular endothelial growth factor receptor signaling	41	0.063	5.80E-18	5.3	3.40E-15
0007173	Epidermal growth factor receptor signaling	38	0.058	1.40E-13	4.3	3.40E-11
0008543	Fibroblast growth factor receptor signaling	35	0.054	8.70E-13	4.4	1.90E-10
0048009	Insulin-like growth factor receptor signaling	4	0.006	5.80E-3	10.4	8.10E-2
0008285	Negative regulation of cell proliferation	34	0.052	1.40E-8	3.1	9.30E-7
0048662	Negative regulation of smooth muscle cell proliferation	8	0.012	2.50E-5	8.9	7.50E-4
0048384	Retinoic acid receptor signaling	7	0.011	3.80E-6	15.1	1.40E-4
0033189	Response to vitamin A	7	0.011	7.90E-6	13.5	2.70E-4
0032526	Response to retinoic acid	8	0.012	2.60E-4	6.2	5.70E-3
0007179	Transforming growth factor-β receptor signaling	15	0.023	1.90E-5	4.1	6.10E-4
0030512	Negative regulation of transforming growth factor-β receptor signaling	9	0.014	9.70E-4	4.4	1.80E-2
0043065	Positive regulation of apoptotic processes	23	0.035	2.50E-5	2.8	7.60E-4
0006915	Apoptotic processes	33	0.051	2.30E-4	2.0	5.00E-3

Benjamini score was derived from the DAVID database. Percentage values were calculated from the associated genes/total genes. GO, gene ontology.

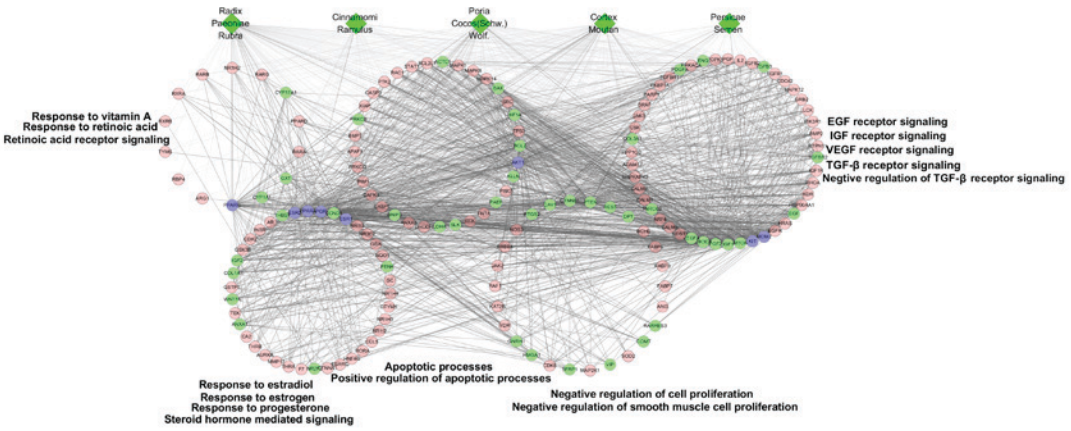

Figure 3. GO enrichment analysis of compound targets, uterine fibroids targets and compound target/uterine fibroids targets. According to the associated biological processes, compound targets of Guizhi Fuling Wan and uterine fibroids targets were related to various molecular mechanisms of uterine fibroids. Green diamonds, pink circles, green circles and blue circles represent the herbs, compound targets, uterine fibroids targets and compound-uterine fibroids targets, respectively; light lines indicate associations between herbs and other nodes; and dark lines indicate associations between compound-uterine fibroids targets and compound targets. EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β.

kinase 2 in signal transduction and oncology (fold enrichment=4.1; P<0.001), platelet-derived growth factor (PDGF) signaling pathway (fold enrichment=3.3; P=0.0016), TGF-β signaling pathway (fold enrichment=2.2; P=0.033), nuclear factor-κB (NF-κB) signaling pathway (fold enrichment=2.1; P=0.043), extracellular signal-regulated kinase (Erk)1/Erk2 MAPK signaling pathway (fold enrichment=2.5; P=0.033; Table II and Fig. 4)

Compound target-uterine fibroids target-other human proteins PPI network analysis. This network contained 2,112 nodes and 67,861 edges (Fig. 5). In this network, nodes with indices higher than the average values (gegree ≥0.0006257, node betweenness ≥0.4363, closeness ≥64.26) were regarded as main nodes. A total of 337 main nodes were selected for GO and pathway enrichment analyses.

Based on GO enrichment analysis, a direct interaction network between the main nodes was established. As depicted in Fig. 6, the main nodes were divided into four functional modules. Module 1 is associated with cell proliferation including negative regulation of cell proliferation (GO ID: 0008285; fold enrichment=4.6; P<0.001) and negative regulation of smooth muscle cell proliferation (GO ID: 0048662; fold enrichment=8.9; P<0.001). Module 2 is associated with the response of cells to steroid hormones including steroid hormone-mediated signaling pathway (GO ID: 0043401; fold enrichment=10.0; P<0.001), response to estrogen (GO ID: 0043627; fold enrichment=9.8; P<0.001) and response to estradiol (GO ID: 0032355; fold enrichment=9.2; P<0.001). Module 3 is associated with apoptosis including positive regulation of apoptotic processes (GO ID: 0043065; fold enrichment=5.6; P<0.001) and the apoptotic signaling pathway (GO ID: 0097190; fold enrichment=4.1; P<0.001). Module 4 is associated with the response of cells to growth factors including VEGF receptor signaling pathway (GO ID: 0048010; fold enrichment=9.3; P<0.001), EGF receptor signaling pathway (GO ID: 0007173; fold enrichment=9.7; P<0.001), FGF receptor signaling pathway (GO ID: 0008543; fold enrichment=10.1; P<0.001), IGF receptor signaling pathway (GO ID: 0048009; fold enrichment=14.0; P=0.0026), TGF-β receptor signaling pathway (GO ID: 0007179; fold enrichment=10.9; P<0.001), negative regulation of the TGF-β receptor signaling pathway (GO ID: 0030512; fold enrichment=7.2; P<0.001). These data are presented in Table III.

Pathway enrichment analysis of the major nodes indicated that the nodes were primarily related to the PI3K-Akt signaling pathway (fold enrichment=4.6; P<0.001), Ras signaling pathway (fold enrichment=5.1; P<0.001), MAPK signaling pathway (fold enrichment=3.8; P<0.001), estrogen signaling pathway (fold enrichment=8.5; P<0.001), ErbB signaling pathway (fold enrichment=9.0; P<0.001), VEGF signaling pathway (fold enrichment=10.7; P<0.001), mTOR signaling pathway (fold enrichment=2.8; P=0.0056), Wnt signaling pathway (fold enrichment=3.6; P<0.001), apoptosis (fold enrichment=4.9; P<0.001), EGF signaling pathway (fold enrichment=5.4; P<0.001), IGF-1 signaling pathway (fold enrichment=5.9; P<0.001), TGF-β signaling pathway (fold enrichment=3.9; P<0.001) and NF-κB signaling pathway (fold enrichment=4.0; P<0.001; Table IV and Fig. 7).

Discussion

At present there is a lack of effective treatment for uterine fibroids. The therapeutic strategies used in western medicine include surgical treatment and pharmacological strategies, though both achieve unsatisfactory outcomes (12-14). TCM recipes exert therapeutic effects on a number of incurable diseases, including uterine fibroids (15). Due to the multi-component and multi-target features of TCM, the research approach for TCM should be different to that of western medicine. However, many studies still apply the conventional research approach of 'one drug, one target, one illness', which does not account for the multi-target and multi-component characteristics of TCM recipes (19-22).

Due to the development of bioinformatics, the network approach has become a novel means of efficiently and

Table II. Pathway enrichment analysis of compound targets, uterine fibroids targets and compound targets/uterine fibroids targets.

Pathway	Gene count	%	P-value	Fold enrichment	Benjamini
Phosphoinositide 3-kinase/ Akt signaling	56	0.086	9.10E-14	3.0	2.70E-12
Ras signaling	44	0.068	1.20E-13	3.6	3.30E-12
MAPK signaling	32	0.049	1.30E-5	2.3	6.80E-5
Estrogen signaling	25	0.038	2.30E-10	4.7	2.80E-9
Vascular endothelial growth factor signaling	22	0.034	2.10E-12	6.7	4.60E-11
Mechanistic target of rapamycin signaling	16	0.025	2.40E-7	5.1	1.60E-6
Wingless-type signaling	14	0.022	3.20E-2	1.9	7.10E-2
Apoptosis	12	0.018	3.90E-4	3.6	1.50E-3
Epidermal growth factor signaling	11	0.017	2.40E-4	3.8	4.50E-3
Insulin-like growth factor-1 signaling	10	0.015	1.30E-4	4.5	3.20E-3
Role of ErbB2 receptor tyrosine kinase 2 in signal transduction and oncology	10	0.015	3.10E-4	4.1	5.30E-3
Platelet-derived growth factor signaling	10	0.015	1.60E-3	3.3	1.70E-2
Transforming growth factor-β signaling	10	0.015	3.30E-2	2.2	7.20E-2
Nuclear factor-κB signaling	10	0.015	4.30E-2	2.1	9.00E-2
Extracellular signal-regulated kinase 1/2-MAPK signaling	8	0.012	3.30E-2	2.5	1.50E-1

Benjamini score was derived from the DAVID database. Percentage values were calculated from the associated genes/total genes.

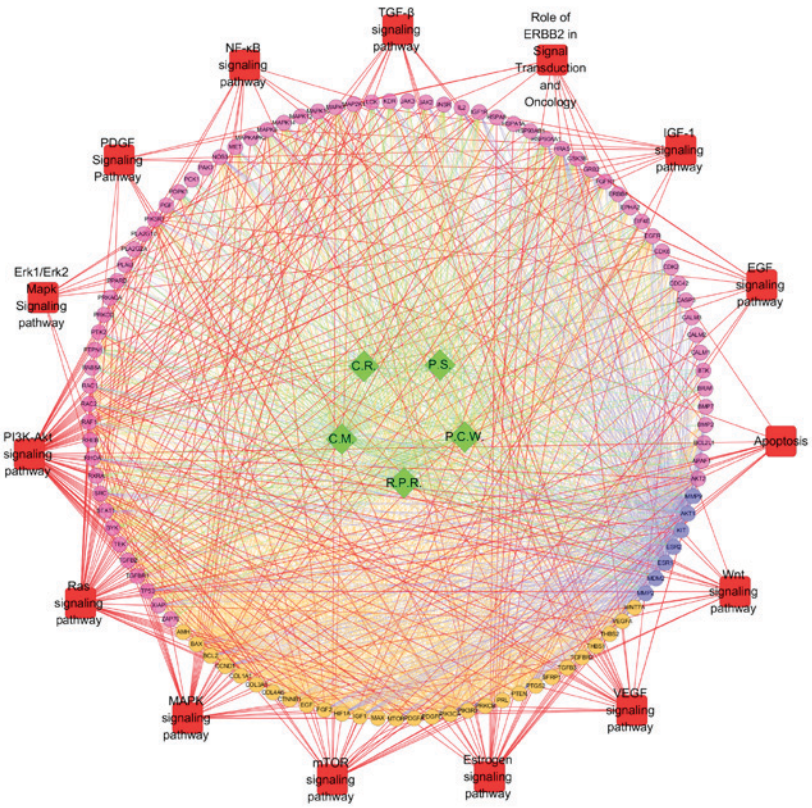

Figure 4. Pathway enrichment analysis of compound targets, uterine fibroids targets and compound targets/uterine fibroids targets. According to pathway enrichment analysis, compound targets of GFW and uterine fibroids targets were related to various pathways. Green diamonds and pink, orange and blue circles represent the herbs, compound targets, uterine fibroids targets and compound-uterine fibroids targets, respectively; Red squares indicate the pathway; red lines indicate the associations between pathways and targets; green lines indicate the associations between herbs and targets; orange lines indicate the associations between uterine fibroids targets and other nodes; and blue lines indicate the associations between compound-uterine fibroids targets and other nodes. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; Erk, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; TGF-β, transforming growth factor-β; IGF, insulin-like growth factor; EGF, epidermal growth factor; Wnt, wingless-type; VEGF, vascular endothelial growth factor; mTOR, mechanistic target of rapamycin; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; C.R., *Cinnamomi Ramulus*; C.M., *Cortex Moutan*; P.S., *Persicae Semen*; P.C.W., *Poria Cocos(Schw.) Wolf.*; R.P.R., *Radix Paeoniae Rubra*.

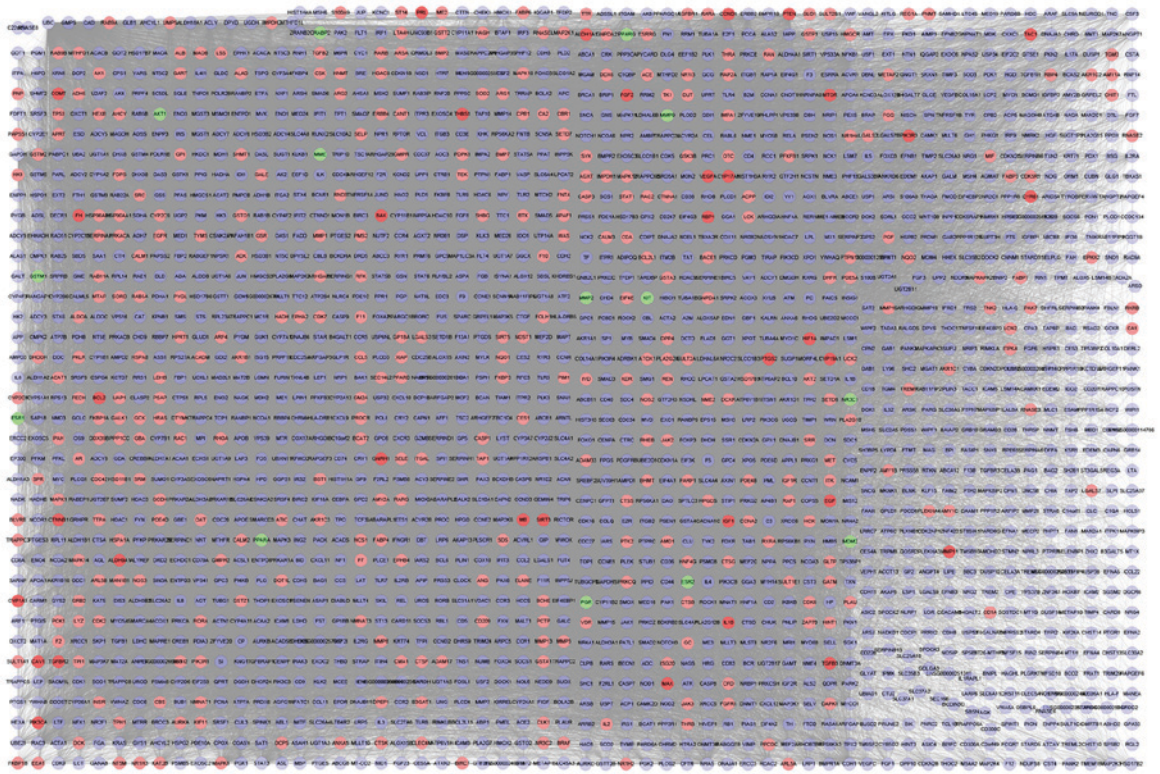

Figure 5. Compound target-uterine fibroids target-other human proteins protein-protein interaction network. Blue, pink, green and red circles indicate the other human proteins, compound targets, compound-uterine fibroids targets and uterine fibroids targets, respectively. The lines represent the nodes associated through protein-protein interactions.

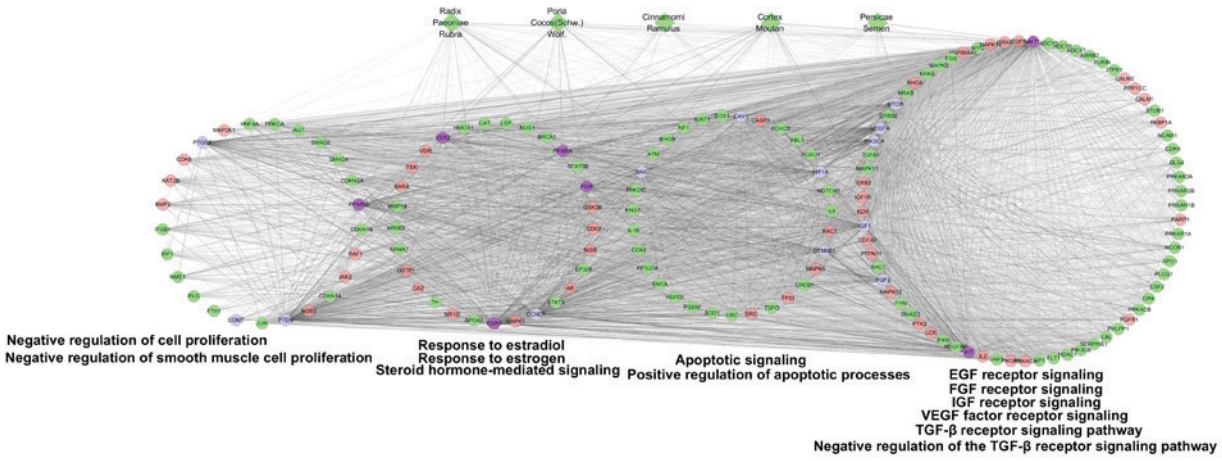

Figure 6. GO enrichment analysis of compound targets, uterine fibroids targets, compound targets/uterine fibroids targets and other human proteins. According to the associated biological processes, the nodes were categorized into four modules. Green diamonds and pink, green, blue and purple circles indicate the herbs, compound targets, other human proteins, uterine fibroids targets and compound-uterine fibroids targets, respectively; light lines represent associations between herbs and other nodes; and dark lines represent associations between compound targets, other human proteins, uterine fibroids targets and compound-uterine fibroids targets. EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor.

systemically identifying the potential molecular mechanisms of TCM recipes. In the present study, a number of network-based computational methods and algorithm-based approaches were used to predict targets and construct networks, in order to assess the molecular interactions associated with GFW when used as a uterine fibroids therapy.

Other studies have demonstrated that there are numerous pathways associated with the development of uterine fibroids (35-42). In particular, steroid signaling (estrogen and progesterone) has been implicated as a key factor in the progression of uterine fibroids (35-38). Alterations in other signaling pathways involving growth factors and their cognate receptors also promote the growth and development of uterine fibroids (39-42).

Regarding the steroid pathway, aberrant and rapid MAPK signaling responses to estradiol may effect on leiomyoma

Table III. GO enrichment analysis of compound targets, uterine fibroids targets, compound targets/uterine fibroids targets and other human proteins.

GO ID	Pathway	Gene count	%	P-value	Fold enrichment	Benjamini
0007173	Epidermal growth factor receptor signaling	64	0.124	5.80E-44	9.7	5.60E-41
0008543	Fibroblast growth factor receptor signaling	60	0.117	4.60E-42	10.1	3.60E-39
0048010	Vascular endothelial growth factor receptor signaling	53	0.103	4.90E-35	9.3	2.40E-32
0048009	Insulin-like growth factor receptor signaling	4	0.008	2.60E-3	14.0	3.00E-2
0007179	Transforming growth factor-β receptor signaling	30	0.058	8.60E-22	10.9	1.70E-19
0030512	Negative regulation of transforming growth factor-β receptor signaling	11	0.021	2.70E-6	7.2	6.10E-5
0032355	Response to estradiol	22	0.043	2.40E-14	9.2	2.10E-12
0043627	Response to estrogen	13	0.025	6.10E-9	9.8	2.20E-7
0043401	Steroid hormone-mediated signaling	12	0.023	2.40E-8	10.0	7.60E-7
0008285	Negative regulation of cell proliferation	37	0.072	5.20E-14	4.6	3.90E-12
0048662	Negative regulation of smooth muscle cell proliferation	6	0.012	5.00E-4	8.9	6.90E-3
0097190	Apoptotic signaling	10	0.019	7.60E-4	4.1	1.00E-2
0043065	Positive regulation of apoptotic processes	34	0.066	2.10E-15	5.6	2.20E-13

Benjamini score was derived from the DAVID database. Percentage values were calculated from the associated genes/total genes.

Table IV. Pathway enrichment analysis of compound targets, uterine fibroids targets, compound targets/uterine fibroids targets and other human proteins.

Pathway	Gene count	%	P-value	Fold enrichment	Benjamini
Phosphoinositide 3-kinase/Akt signaling	73	0.142	1.30E-29	4.6	3.80E-28
Ras signaling	53	0.103	2.20E-23	5.1	3.30E-22
Mitogen-activated protein kinase signaling	45	0.087	7.20E-15	3.8	4.40E-14
Estrogen signaling	39	0.076	4.60E-26	8.5	9.90E-25
ErbB signaling	36	0.070	6.90E-25	9.0	1.40E-23
Vascular endothelial growth factor signaling	30	0.058	2.60E-23	10.7	3.70E-22
Wingless-type signaling	23	0.045	2.30E-7	3.6	7.20E-7
Epidermal growth factor signaling	20	0.039	1.70E-11	5.4	8.20E-10
Insulin-like growth factor-1 signaling	17	0.033	1.10E-10	5.9	3.90E-9
Nuclear factor-κB signaling	16	0.031	8.10E-6	4.0	2.20E-5
Transforming growth factor-β signaling	15	0.029	2.10E-5	3.9	5.40E-5
Apoptosis	14	0.027	3.50E-6	4.9	9.70E-6
Mechanistic target of rapamycin signaling	10	0.037	5.60E-3	2.8	2.30E-2

Benjamini score was derived from the DAVID database. Percentage values were calculated from the associated genes/total genes.

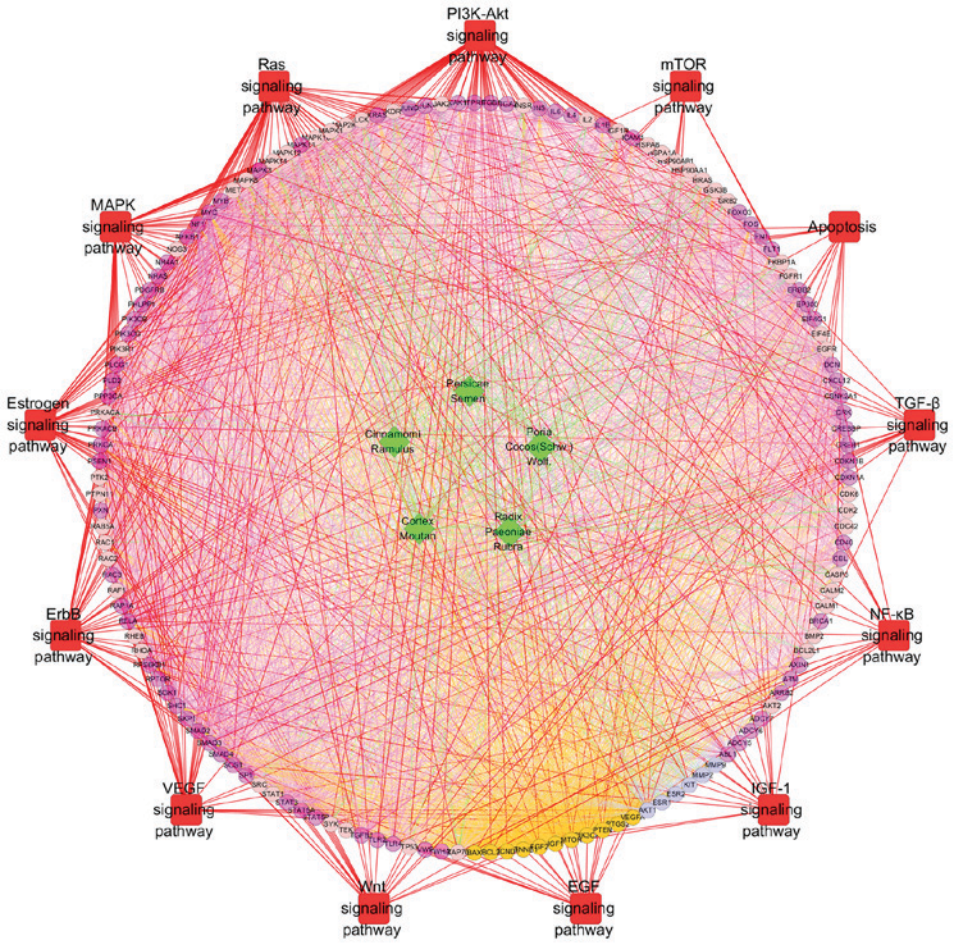

Figure 7. Pathway enrichment analysis of compound targets, uterine fibroids targets, compound targets/uterine fibroids targets and other human proteins. Pathway enrichment indicated that the major nodes were primarily linked to the indicated pathways. Green diamonds and light pink, dark pink, orange and blue circles indicate the herbs, compound targets, other human proteins, uterine fibroids targets and compound-uterine fibroids targets, respectively; red squares indicate the pathways; red lines indicate the associations between pathways and targets; green lines indicate the associations between herbs and targets; orange lines indicate the associations between uterine fibroids targets and other nodes; and blue lines indicate the associations between compound-uterine fibroids targets and other nodes. PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; mTOR, mechanistic target of rapamycin; TGF-β, transforming growth factor-β; NF-κB, nuclear factor-κB; IGF, insulin-like growth factor; EGF, epidermal growth factor; Wnt, wingless-type; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase.

proliferation (43). It has been observed that compared with the surrounding myometrium, the mRNA transcription of estrogen receptor (ER)α and ERβ is elevated in leiomyoma tissue (44-45). Maekawa *et al* (46) demonstrated that epigenetic regulation of ERα through DNA methylation may serve a role in leiomyoma. In addition, it has been reported that progresterone as well as estrogen may serve a significant role in leiomyoma development (38). Compared with estrogen-mediated myometrium proliferation during the menstrual cycle, secretion of progesterone promotes mitotic activity in uterine leiomyomas (35,38). Furthermore, leiomyoma xenograft animal models have indicated the necessity of progesterone for the growth of uterine leiomyoma (47). Progesterone-bound progesterone receptor (PR) can not only accelerate the transcription of specificity protein-1 (SP-1) (35), as a transcription factor itself, but may also activate signaling pathways. For instance, ligand-bound PRs may activate protein kinases involved in growth factor signaling, such as MAPK and MEK (48).

The role of progesterone in the development of uterine fibroids is complex. Estradiol may induce an upregulation in PRs in leiomyoma cells (47), and interactions between progesterone and growth factor signaling may also promote the development of leiomyoma. For instance, progesterone may downregulate the expression of IGF-1 in human leiomyoma cells (49), upregulate the expression of proliferating cell nuclear antigen (PCNA) and EGF, as established regulators of leiomyoma cellular proliferation (50,51), and activate the Akt pathway to mediate leiomyoma proliferation (4). These findings suggest that progesterone signaling is involved in complex signaling networks associated with leiomyoma.

Regarding growth factors, previous studies suggest that alterations in certain growth factors and their cognate receptors or signaling pathways serve a significant role in the growth and development of uterine fibroids (39-42). These factors include IGF-1 (52,53), PDGF (42), VEGF (54), EGF (55), and FGF (56). Activation of receptor tyrosine kinases (RTKs) is a critical biological process; growth factor binding to RTKs leads receptor dimerization and autophosphorylation, thereby activating the downstream pathways Grb2/Sos/Ras/Raf/MEK/Erk and PI3K/PIP3/Akt to regulate proliferation, differentiation, survival and metabolism (57,58). Previous results have indicated that IGF-1 signaling is regulated by estrogen, and that 17β estradiol treatment leads to increases in IGF-1 mRNA and Myb, a transcription factor that promotes the expression of cell cycle progression genes in leiomyoma cells (59). Furthermore, estradiol has been demonstrated to promote the upregulation of growth factors and RTKs in uterine leiomyomas, indicating that growth factors and RTKs represent intermediate effectors of sex steroids in leiomyomas (60). It has also been demonstrated that under the influence of estrogen, the Ras/Raf/MEK/ERK signaling pathway (43,59) and PI3K/Akt/mTOR signaling pathway (57,61,62), activated by RTK-ligand complexes, exert significant effects on the pathological growth and development of fibroids. Expression of the Smad signaling pathway mediated by ligands including TGF-β, activin, mystatin, BMP and others belonging to the TGF-β superfamily is also associated with leiomyoma, and is now becoming a potential therapeutic target (63). Furthermore, compared with normal smooth muscle cells, IGF-2 mRNA is upregulated in uterine leiomyoma samples, and levels of IGF-1 are associated with Akt activation (53). In uterine fibroids, both EGF and PDGF have been found to stimulate protein synthesis in leiomyoma and myometrial cells (64). Notably, downstream signaling induced by EGF stimulation is altered in leiomyoma cells when compared with myometrial cells (65).

VEGF was an essential factor for the growth of leiomyoma xenografts *in vivo* (66-68). Furthermore, its cognate receptors VEGFR-1 and VEGFR-2, and VEGF-A, were significantly overexpressed in leiomyoma when compared with adjacent myometrium (66-68). A recent study on Wnt signaling in the growth and development of uterine fibroids demonstrated that the Wnt/β-catenin signaling pathway mediated a novel interaction between leiomyoma stem cells (representing 1% of tumor cells; also known as a leiomyoma side-population), and mature leiomyoma cells, which promoted tumor growth (69). Furthermore, the paracrine effects of estrogen and progesterone may stimulate leiomyoma cells to proliferate through Wnt/β-catenin signaling (69). As an additional factor, RA is an active metabolite of vitamin A (retinol) and primarily promotes cellular growth and development (70). Previous results suggest that receptors of RA signaling (RA and retinoid X receptors) are expressed in leiomyoma cells (70).

The characteristics of fibroids are principally due to the clonal proliferation of single smooth muscle cells in the myometrium and alterations in complex signal pathways (3,4). The associated signaling pathways are mediated by multiple factors, including steroids, growth factors, TGF-β/Smad, Wnt/β-catenin and RA (3,4). These signaling molecules in leiomyoma cells serve a common role of mediating secretion from peripheral stromal cells to regulate leiomyoma growth (3,4). The regulation of estrogen and progesterone signaling may be an important method for treating uterine fibroids. For instance, continuous administration of gonadotropin-releasing GnRHa induced menopausal status and lowered estrogen level, which was associated with tumor shrinkage (71). However, due to the side effects of long-term GnRHa use, including loss of bone mineral density (71), it may only be used for a relatively short period. Progesterone antagonist and selective progesterone receptor modulator (SPRM) may inhibit proliferation and induce apoptosis in leiomyoma cells (72,73). Furthermore, SPRM was able to reduce the expression of IGF-1 (43), VEGF (74), EGF and TGF-β (75), which inhibits estrogen and progesterone signaling, and the Ras/Raf/MEK/Erk and PI3K/Akt/mTOR signaling pathways (75). Asoprisnil, a SPRM, decreased the expression of certain growth factors and growth factors receptors in leioyoma, including EGFR, IGF-1Rα and TGFRII (75). However, its side effects included endometrial hyperplasia and breast pain and discomfort (76).

The TGF-β/Smad and RA signaling pathways also represent potential targets for therapeutic development. Previous data indicate that GnRHa may decrease the expression of TGF-β receptors, Smad4 and phosphorylated Smad3 (63), and all-trans RA may inhibit the proliferation of leiomyoma cells (77). At present, the major therapeutic agents for uterine leiomyomas are steroids, SPRMs and selective estrogen receptor modulators. However, long-term usage might cause reproductive system side effects (12-14). The present

experimental data demonstrated that uterine fibroids may also be alleviated by GFW through its downregulatory effects on estrogen, progesterone and their cognate receptors and production of TGF-β/Smad.

In uterine fibroids, the upregulation of five key factors, namely steroids, growth factors, TGF-β/Smad, Wnt/-catenin and RA, is closely related with tumor growth and development (4). Leiomyoma and peripheral stromal cells cooperate to promote leiomyoma proliferation and increase the synthesis of peripheral matrix proteins (proteoglycans and fibronectins) by paracrine signaling, which forms a complex network involving alterations in cell shape and cytoskeleton (78,79). Thus, regulation of paracrine signaling molecules, as a therapeutic strategy for uterine fibroids, may inhibit the synthesis of matrix proteins and proteoglycans, inhibit the proliferation of leiomyoma cells and promote leiomyoma cell apoptosis (4). Proliferation-related signaling pathways mediated by estrogen and progesterone serve complex and important roles in the pathology of uterine fibroids (39). Thus, use of estrogen and progesterone antagonists, including GnRHa and synthetic steroids, with progesterone antagonists such as mifepristone and asoprisnil, may effectively inhibit the development of leiomyoma, and warrants further study. Future research should also focus on the potential regulatory effects of GFW on estrogen and progesterone receptors.

Cell apoptosis, particularly of leiomyoma, peripheral stromal and fibroids vascular endothelial cells and leiomyoma stem cells, has been closely associated with fibroids development (4). Leiomyoma stem cells serve roles in the organizational structure and generation of leiomyoma cells, and peripheral matrix synthesis, which contributes to the development of fibroids (4). Thus, inducing the apoptosis of various types of leiomyoma cells is a terminal method for treating uterine fibroids. Previous studies have demonstrated that total paeony glucosides, including paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, benzoylalbiflorin and albiflorin, may induce tumor cell apoptosis by increasing intracellular $Ca2^{+}$ concentration, inhibiting the mRNA transcription of B-cell lymphoma 2 (Bcl-2), Bcl-extra large and upregulating Bcl-2-associated X protein expression (79,80). Furthermore, human leiomyoma cell proliferation may be inhibited by paeonol, pachymic acid, albiflorin and paeoniflorin (80,81). It has also been reported that GFW may promote tumor cell apoptosis and inhibit human leiomyoma cell proliferation (81,82). Therefore, GFW may prevent the growth of uterine fibroids by promoting tumor cell apoptosis.

The uterine fibroids are characterized by excessive deposition of extracellular matrix and proliferation of fibroids, which is mediated by a variety of signaling pathways (4). For instance, receptor-bound growth factors, estradiol and progesterone can activate Ras/Raf/MEK/Erk, and thus inhibition of Ras/Raf/MEK/Erk may be an effective therapeutic strategy, indicating the importance of double- and multi-target treatments. At present, therapeutics that regulate multiple targets and pathways are regarded as an alternative method in the management of cancers such as prostate cancer (83). GFW may exert therapeutic effects against uterine fibroids through multi-pathway and -target activity.

According to the current predictions based on network pharmacology, a number of novel signaling pathways and biological processes underlying the effects of GFW on uterine fibroids were identified. The results also provided a rationale for the combination of herbs within GFW. This network pharmacology method may aid the systematical study of herbal formulae and make TCM drug discovery more predictable. Evaluating the efficacy of TCM recipes and identifying the corresponding pharmacological mechanism on a systematic level may be a useful method for future studies.

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