Assessing the Anti-cancer Therapeutic Mechanism of a Herbal Combination for Breast Cancer on System-level by a Network Pharmacological Approach

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Abstract. *Background/Aim:* Accumulating evidence has shown therapeutic effects of herbals on breast cancer, a commonly diagnosed malignancy in women worldwide. However, their underlying mechanisms remain unclear. We aimed to explore the mode of action of a recently developed herbal combination at system-level. *Materials and Methods:* We employed network pharmacological approaches to study the mechanism of a combination of three herbals, *Astragalus membranaceus*, *Angelica gigas* and *Trichosanthes kirilowii* by investigating active compounds and performing functional enrichment analysis for the interacting targets. *Results:* For *in silico* pharmacokinetic evaluation, ten active ingredients interacted with fifty-six breast cancer-associated therapeutic targets. Functional enrichment analysis revealed that TNF, estrogen, PI3K-Akt and MAPK signaling pathways were involved in tumorigenesis and development of breast cancer. The pharmacological mechanisms might be associated with cellular effects on proliferation, cell cycle process and apoptosis. *Conclusion:* The present study provides novel insights into the system-level pharmacological mechanisms underlying a herbal combination used for breast cancer therapies.

Breast cancer (BC) is the most commonly diagnosed female malignancy throughout women's life cycle (1). A growing body of evidence has demonstrated that BC is a multifactorial disease that is driven by a variety of genetic and epigenetic alterations and the subsequent dysregulation of diverse

oncogenic signaling pathways (2). Recent advances in the elucidation of the molecular mechanisms underlying cancer progression have identified that the dysregulated pathways include estrogen, phosphatidylinositol 3-kinase (PI3K)-Akt, mitogen-activated protein kinase (MAPK), vascular endothelial growth factor receptor (vEGFR), Janus kinase (Jak)-signal transducer and activator of transcription (STAT), erythroblastic leukemia viral oncogene homologue (ErbB) and p53 signaling pathways (3-5). Current standard pharmacological care for BC therapies include conventional cytotoxic chemotherapy and molecular targeted therapy which aim to modulate the aberrant activation of these diverse oncogenic pathways (6). However, the use of cytotoxic chemotherapeutic agents has been reported to cause unwanted effects that adversely affect the quality of life (QoL) of cancer patients (7). Furthermore, the targeted anticancer drugs presently used in clinics are designed based on the 'one drug-one target' paradigm and therefore frequently have limited efficacy (8, 9). These issues underscore the importance of the development of anti-cancer therapies that can effectively target multiple oncogenic pathways with improved safety.

Meanwhile, herbals and their derived biochemical compounds are being recognized as effective therapeutic agents for cancer therapies (10, 11). Herbals, characterized by multiple compounds that affect multiple targets and pathways, have attracted much attention since they have been reported to possess potent anticancer properties through the modulation of multiple pathways relevant to the initiation and progression of cancer (12). An increasing number of clinical studies have also indicated their beneficial roles on improving QoL of cancer patients (7).

SH003 is the code name of a recently developed herbal combination that consists of three herbals, *Astragalus membranaceus* (Am), *Angelica gigas* (Ag) and *Trichosanthes kirilowii* (Tk) and has been shown to exert anticancer effects (13-21). It has been reported to efficiently suppress proliferation, growth, metastasis and angiogenesis. It has also been shown to induce apoptosis of various types of cancer cells including BC cells, *in vitro* and *in vivo* (22). Although

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the herbal combination has been shown to exhibit therapeutic activity in part by inhibiting key pro-tumorigenic pathways, such as STAT3/interleukin (IL)-6, VEGFR2 and extracellular signal-regulated kinase (ERK) signaling, its system-level pharmacological mechanism in BC has not been fully investigated yet.

Network pharmacology is an emerging interdisciplinary strategy that aims at the understanding of the underlying pharmacological mechanisms of herbal combinations from a systems perspective. This methodology has been widely applied to discover the major active compounds of herbals and their corresponding potential targets. It is also helpful in investigating the synergistic, additive, antagonistic or poly-pharmacological properties generated from the complicated interactions of multiple compounds and relevant targets at a complex network-level, which facilitates the elucidation of the modes of action of herbals (12, 23, 24). In this study, we attempted to explore the therapeutic mechanism of the herbal combination SH003 for BC therapies at the systems-level using a network pharmacological approach.

Materials and Methods

Investigation on the chemical compounds. The chemical compounds of the three herbals Am, Ag and Tk, were obtained from traditional Chinese medicine (TCM) databases, such as, Traditional Chinese Medicine Systems Pharmacology (TCMSP) database, Herb Ingredients' Targets (HIT) database, Traditional Chinese Medicine Integrated Database (TCMID) and further manually supplemented through an extensive search of various articles (25-28). Note that in the case of Ag, we first retrieved the chemical components contained in *Angelica gigas*, a herbal of the *Angelica* genus widely used in China using the abovementioned TCM databases and integrated these results with the previously reported major compounds that are found in Ag, including decursin, lomatol, and marmesin. Moreover, the chemical compounds that are not present in Ag, such as, 4-octanone, isoeugenol and stigmasterol were omitted from the collected data based on previous experimental results (29-31).

Active compound screening. To identify the potential bioactive compounds of the SH003 herbal combination, we investigated the absorption, distribution, metabolism and excretion (ADME) profiles of each chemical compound derived from the three herbals, Am, Ag and Tk. In this study, the three most widely used pharmacokinetic parameters oral bioavailability (OB), Caco-2 permeability and drug-likeness (DL) were evaluated using the TCMSP database (28). OB refers to the fraction of an orally administered dose that passes through the gastrointestinal epithelium towards the systemic circulation, and is detectable to targeted internal tissues and organs (28, 32). Caco-2 permeability is a widely applied index to predict the intestinal absorption of potential of drug molecules and chemical compounds, which is determined by measuring the rate of their diffusion mobility across the Caco-2 human large intestinal epithelial cancer cell line (28, 33, 34). In general, chemical compounds whose Caco-2 permeability is less than -0.4 are considered to be impermeable (35). DL is a qualitative index used to assess whether a prospective compound is chemically suitable for

drug design based on its molecular properties and structural features (28). Chemical compounds without sufficient ADME information were excluded from the screening process and those with OB $\geq$ 30%, Caco-2 permeability $\geq$ -0.4 and DL $\geq$ 0.18 were considered to be pharmaceutically active compounds in each herbal, as suggested by previous studies (28, 36, 37).

Investigation on target proteins interacting with the compounds. Human proteins that interact with the identified active compounds within the herbal combination were determined using the Search Tool for Interactions of Chemicals (STITCH) 5 database (38). The proteins interacting with the active compounds with confidence scores $\geq$ 0.7 (a reference score for a high-confidence association as indicated by STITCH 5) under the 'Homo sapiens' species setting were considered as targets of the herbal combination. Human target genes and proteins of individual active compounds were also investigated using HIT and Therapeutic Target Database (TTD) databases (39). Information on target proteins including the name, protein ID and organism, was further confirmed using Uniprot (40). List of proteins associated with tumorigenesis and development of BC were obtained from various databases such as GeneCards (41), TTD (39), Online Mendelian Inheritance in Man (OMIM) (42), DrugBank (43), Pharmacogenomics Knowledge for Personalized Medicine (PharmGKB) (44), and DisGeNET (45) by using the search term, 'breast cancer' with search species limited to 'Homo sapiens'.

Network construction. The herb-compound (H-C) network was built by connecting the herbs with their active compounds, and the compound-target (C-T) network was constructed by linking the active compounds with their corresponding target proteins. The target-pathway (T-P) network was generated by the linkage of the target proteins and their related signaling pathways. All the networks were visualized by Cytoscape software (version 3.6.1) (46). In the networks, the nodes represent the herbs, active compounds, target proteins or signaling pathways and edges represent the interactions between the nodes.

Functional enrichment analysis. The functional enrichment analysis of distinct sets of target proteins was performed using g: Profiler, a web server-based tool used for the functional analysis of genes or proteins, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (47, 48).

Calculation of contribution indices. To evaluate the contribution of individual active compounds to the anti-cancer effects of the herbal combination, a contribution index (CI) was calculated based on the network-based efficacy (NE) according to the following equations [1] and [2] as previously described (37, 49):

$$NE(j) = \sum_{i=1}^n d_i \quad [1]$$

$$CI(j) = \frac{c_j \times NE(j)}{\sum_{i=1}^m c_i \times NE(i)} \times 100\% \quad [2]$$

where n is the number of proteins targeted by compound j; d_i is the degree of protein i targeted by compound j; m is the number of compounds and CI is the number of studies relevant to both BC and compound i . To survey the literature correlated with BC and the active compounds, the term 'breast cancer' and the common names

Figure 1. A schematic illustration representing the workflow for the network pharmacological analysis to reveal the therapeutic mechanisms of the herbal combination.

of compounds were used as search keywords. The number of articles published from 1990 to 2019 containing the aforementioned keywords in the abstract were obtained from PubMed. If the sum of the CIs for the top N compounds was greater than 85%, these N compounds were considered the major contributors to the anti-cancer effects of the herbal combination as previously described (37, 49).

Results

The pharmacological mechanisms of the herbal combination against BC were explored at the systems-level. First, the chemical compounds of the herbals were collected from TCM-related databases. Next, the pharmacokinetic parameters including OB, Caco-2 cell permeability, and DL, of individual compounds were evaluated to identify the potential bioactive compounds. Then, the human proteins targeted by the active compounds were determined *via* the investigation of the protein-chemical interaction data and functional enrichment analysis of the target proteins was performed. Afterwards, the comprehensive pharmacological information associated with the herbal combination was integrated into the H-C, C-T, and T-P networks and the underlying therapeutic mechanisms of the herbal medicine were analyzed (Figure 1).

Chemical compounds. The chemical compounds of the three herbals Am, Ag and Tk, were retrieved from a number of TCM databases (*e.g.*, TCMSP, TCMID and HIT) and relevant literature (25-27, 37, 49). As a result, 113, 154, and

80 compounds were retrieved for Am, Ag and Tk, respectively; 14 of those compounds were duplicates. Hence, a total of 331 compounds were identified after removing the duplicate phytochemicals.

Screening of the active phytochemical compounds. The *in silico* ADME system has been useful for the evaluation and screening of potent chemical compounds that may exhibit druggable pharmacokinetic activity (28, 37). As a result, 33 compounds were identified as active phytochemical compounds.

Investigation of the target proteins of the active compounds. We employed an *in silico* approach and analyzed the interactions between the herbal active compounds and proteins using the STITCH 5 database (38). As a result, we identified a total of 117 target proteins for the herbal combination (Figure 2).

Network-based investigation of the therapeutic profiles. To visualize the ‘multi-compound, multi-target’ bioactivity, we constructed herb-compound-target (H-C-T) network. Notably, in the network constructed in the present study, the nodes represent the herbals, active compounds, target proteins, or signaling pathways, and the edges represent the interactions between the nodes. The H-C-T network for the herbal combination consists of 130 nodes and 183 edges, including three herbals, 10 active compounds and 117 target proteins.

Figure 2. The herb-compound-target network. Green hexagons indicate the three herbal medicines comprising the herbal combination and red rectangles indicate their 10 active chemical compounds. Ovals represent the 117 targets of the active compounds of SH003, where those associated with breast cancer are colored in blue.

To further explore their systems-level pharmacological features based on a network perspective, a C-T network comprising 66 nodes and 89 edges was generated by linking the screened active compounds with their targets that are closely associated with BC (Figure 3). Note that the centralization and heterogeneity of the network were 0.411 and 2.163, respectively. Among the 56 BC-associated targets of the herbal combination, AKT1 (degree=5), caspase-3

(CASP3; degree=5), CYP1A1 (degree=5) and CYP1B1 (degree=5) had the highest degree, indicating that they may act as important hub targets of the pharmacological activities of the herbal combination for BC therapy. These targets have been reported to be closely related to the pathogenesis and development of BC; AKT1 promotes the growth and tumorigenesis of BC cells *in vitro* and *in vivo* (50). The activity of caspase-3 may function as a potential determinant

Figure 3. The compound-target network. Red rectangles indicate the 10 active chemical compounds in the herbal combination and blue ovals indicate their 56 breast cancer-associated targets.

of the survival or apoptosis of BC cells and tumors (51) and its expression levels have been associated with the survival rate of BC patients (52). CYP1A1 and CYP1A2, extrinsic pathways of the cytochrome P450 (CYP) superfamily, are key enzymes implicated in estrogen metabolism and may play important roles in the development, survival, proliferation and progression of BC (53, 54).

To assess the contribution of individual active compounds to anticancer effects, the CI was calculated for every active compound based on NE as previously described (47). As a result, three compounds including quercetin, kaempferol and vitamin E were found to have high CIs with a sum of 89.42% (Figure 4).

Functional enrichment analysis of the herbal combination-associated network. To investigate the biological characteristics

Figure 4. Analysis of the contribution index of individual active chemical compounds in the herbal combination. The sum of contribution indices for the top three active compounds, including quercetin, kaempferol, and vitamin E, was more than 85%.

Figure 5. The Herb-Compound-Target-Pathway network. Green hexagons indicate the three herbs comprising the herbal combination and red rectangles indicate their 10 active chemical compounds. Blue ovals indicate the breast cancer-associated targets of the active compounds and orange diamonds indicate the signaling pathways enriched with the corresponding targets.

of the herbal combination-associated network, we performed gene ontology (GO) enrichment analysis of the BC-associated targets that interact with the active compounds of the herbal combination using g: Profiler (47). As a result, we found that the targets were significantly enriched for the genes/proteins associated with a variety of biological processes such as the positive regulation of cell death, apoptotic process or cell cycle arrest and negative regulation of cell proliferation and cell cycle process.

The aberrant regulation of diverse oncogenic signaling pathways is closely implicated in the tumorigenesis of various human cancers. To elucidate the underlying pharmacological mechanisms at the pathway level, we further conducted pathway enrichment analysis of its BC-associated targets based on the KEGG database. As a result, we found that the 'Pathways in Cancer' exhibited the highest number of target connections (degree=33), followed by the 'TNF signaling pathway', 'Estrogen signaling pathway',

‘Breast cancer’ and ‘PI3K-Akt signaling pathway’ with 13 targets, and ‘MAPK signaling pathway’ with 12 targets (Figure 5).

Furthermore, the functional association of the BC-related targets of the herbal combination was analyzed using GeneMANIA, which facilitates the analysis and the prediction of functional interactions between multiple genes/proteins by integrating diverse types of biological information and data (55). The GeneMANIA analysis results demonstrated that among the BC-related targets of the three herbals, 32.33% were predicted to be co-expressed and 29.34% were predicted to have physical interactions.

Discussion

In this study, we attempted to identify the therapeutic mechanisms of action of a newly developed anticancer herbal combination for BC, from a systems-perspective using network pharmacological approaches. Our novel findings obtained from this network analysis-based investigation led to the following conclusions. (i) Ten potential active compounds may interact with 56 BC-related targets to exert therapeutic actions. (ii) The targets interacting with the active compounds are enriched for genes/proteins associated with a variety of biological processes, including positive regulation of cell death, apoptotic process, or cell cycle arrest and negative regulation of cell proliferation or cell cycle process. (iii) The targets are further enriched in multiple pathways in tumorigenesis and development of BC.

The network pharmacological analysis identified 10 active compounds that may interact with 56 BC-related targets. These active compounds, including isorhamnetin (56), kaempferol (57), diosmetin (58), quercetin (59), mairin (betulinic acid) (60), decursin (61), formononetin (62), β -sitosterol (63) and calycosin (64) have been reported to exhibit antitumor activities in BC. Isorhamnetin is known to inhibit cell proliferation and induce apoptosis in human BC *via* the regulation of MAPK and PI3K/Akt pathways (56). Moreover, kaempferol, diosmetin, mairin and quercetin have been shown to function by inducing cell cycle arrest of human BC cells (57, 58, 65, 66). Decursin inhibits Pin1 activation and promotes its association with p53 (67). Together, these findings reveal the chemical basis of the pharmacological effects of the herbal combination on BC therapy. Note that among the active compounds, quercetin, kaempferol and vitamin E may be the major contributors to the pharmacological effects of the herbal combination based on the CIs analysis.

Previous studies have reported that the herbal combination suppresses the growth and metastasis of human BC cells by inhibiting the activity of the STAT3/IL-6 axis (17). They can also modulate the activities of key oncogenic signaling

pathways *in vitro* and *in vivo*, including ERK, VEGF and PI3K/mTOR pathways, which may lead to the induction of cell cycle arrest, apoptosis and antiangiogenic processes in various types of cancer cells (22). These experimental observations are consistent with the network pharmacology-based analysis results (Figure 4). Further experimental studies are warranted to validate the pharmacological mechanisms of the herbals.

In summary, by employing network pharmacological approaches, we explored the underlying pharmacological mechanisms of the SH003 herbal combination from a systems-perspective. We found that the targets interacting with the active compounds were functionally enriched in multiple pathways that are closely associated with tumorigenesis and development of BC, including the TNF signaling pathway, estrogen signaling pathway, PI3K-Akt signaling pathway and MAPK signaling pathway.

Conflicts of Interest

The Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

Conceptualization, HSL and DSO; methodology, HSL and DSO; software, HSL; validation, HSL and DSO; formal analysis, HSL; data curation, HSL and DSO; writing the first draft, HSL; drafting, DSO; supervising, DSO; funding acquisition, DSO.

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