Network Pharmacology Validation of Therapeutic Mechanisms of Tanshinone IIA in Colorectal Cancer

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Abstract
Curative therapies with fewer adverse effects are required for cancer treatment. Medicinal plants represent a promising source of novel therapeutic candidates. We employed network pharmacology to predict potential molecular mechanisms of salvia root-derived tanshinone IIA (Tan IIA) in the treatment of colorectal cancer (CRC), followed by empirical validation. The Traditional Chinese Medicine System Pharmacology (TCMSP), DrugBank, and GeneCards databases were queried to identify overlapping Tan IIA (therapeutic)- and CRC (disease)-relevant protein targets. Cytoscape and STRING were used to generate component-target and protein-protein interaction (PPI) networks, respectively, and topology analysis identified highly connected nodes within the latter. Target proteins were subjected to gene ontology (GO)-based biological process annotation using DAVID, and to biological pathway enrichment analysis using the Kyoto encyclopedia and genome (KEGG) database. Enriched biological processes included cell cycling and proliferation, and enriched KEGG pathways included neuroactive ligand-receptor interaction, PI3K-Akt, and cancer. Network pharmacology results predicted that Tan IIA impacts multiple targets and pathways, but that its therapeutic effect is predominantly attributable to cell cycle regulation, inhibition of cell proliferation, and induction of apoptosis. Investigation of the in vitro impact of Tan IIA on proliferation, viability, and cell cycling of 2 human CRC cell lines (SW480 and SW620), using the CCK-8 method and flow cytometry, demonstrated that Tan IIA significantly inhibits cell proliferation via inducing cell cycle arrest in the G2/M phase. Network pharmacology-predicted hypotheses were thus empirically validated, providing a basis for in-depth study of the therapeutic mechanisms of Tan IIA in the context of CRC.

Keywords
tanshinone IIA, colorectal cancer, network pharmacology, cell cycle, cancer cell proliferation

Received: February 24th, 2021; Accepted: March 1st, 2021.
The dried root and rhizome of red-rooted salvia (Salvia miltiorrhiza Bunge) are known to relieve pain, promote circulation, calm the heart, and heal carbuncles. Individually, chemical compounds within salvia root exhibit extensive anti-neoplastic effects, with tanshinone IIA (Tan IIA) representing the major bioactive constituent. The chemical structure of Tan IIA is shown in Figure 1. The compound is able to inhibit tumor cell proliferation, induce tumor cell apoptosis, modulate tumor cell invasive and metastatic capability, and oppose tumor cell multi-drug resistance. More specifically, Tan IIA regulates CRC tumor cell proliferation, as well as inhibiting tumor angiogenesis (thereby opposing metastasis and recurrence); however, specific therapeutic molecular mechanisms of Tan IIA in the setting of CRC remain largely unknown.

Falling under the purview of Systems Biology, the network pharmacology approach combines traditional pharmacology, network theory, bioinformatics, and biological network analysis to facilitate more comprehensive investigation of the potential molecular mechanisms of medicinal (including plant-derived) compounds. It employs biological network construction, visualization, and analysis to identify the complex interactions between compounds and their disease-relevant targets. The present study uses network pharmacology to explore the potential therapeutic molecular mechanisms of Tan IIA in the context of CRC, first predicting potential protein targets, biological processes, and biological pathways impacted by Tan IIA, followed by empirical validation via investigation of the in vitro effects of Tan IIA on human CRC cell lines (SW480 and SW620) proliferation and viability, as well as cell cycling, using the CCK-8 method and flow cytometry, respectively. The experimental design is shown in Figure 1. To the best of our knowledge, this is the first time network pharmacology (in conjunction with empirical validation) has been used to evaluate the potential therapeutic mechanisms of Tan IIA in the context of CRC.

Materials and Methods

Acquisition of Potential Tan IIA Therapeutic Targets

The search term “Tanshinone IIA” was used to query the Traditional Chinese Medicine System Pharmacology (TCMSP) database (http://lsp.nwu.edu.cn/tcmsp.php) in order to obtain a list of Tan IIA (therapeutic) protein targets. The resultant list of predicted targets was provided as PubChem IDs, which were converted to simplified molecular-input line entries (SMILEs) in order to query the SwissTargetPrediction database (http://www.swistargetprediction.ch). Duplicates were removed from the list.

Acquisition of Potential CRC Disease Targets

The search terms “colorectal cancer” and “colorectal carcinoma” were used to query the DrugBank (https://www.drugbank.ca) and GeneCards (https://www.genecards.org) databases, as well as GEO database [GSE44076, included 246 samples (100 patients and 146 healthy controls) and GSE42284, whole genome expression profile of colorectal cancer, including 188 patient samples] in order to obtain a list of CRC (disease) protein targets. The resultant list of predicted targets was inputted into the UniProt (http://www.uniprot.org/) database to obtain current correct corresponding gene names and Uniprot IDs. Duplicates were removed from the list. Finally, therapeutic and disease target lists were compared by means of a Wayne diagram to identify targets shared between the 2 lists.

Construction of the Component-Target Network

Using Cytoscape plugin version 3.2.1 (http://www.cytoscape.org), a comprehensive “component-target” network was constructed to visualize all known relationships between shared therapeutic and disease targets, facilitating deep analysis of the potential therapeutic mechanisms of Tan IIA in CRC.

Construction of the Protein-Protein Interaction (PPI) Network

Similarly, using the STRING database (https://string-db.org; species: Homo sapiens), a PPI network (confidence score >0.95) was generated to visualize all known relationships between shared therapeutic and disease targets. Proteins independent of (not connected to) the network were excluded, and network topology analysis was applied to identify proteins with high degrees of connectedness.

Functional Annotation and Pathway Enrichment Analysis

Finally, using the DAVID platform (https://david.njcfcrf.gov/), shared therapeutic and disease targets were subjected to gene ontology (GO) functional annotation (at the level of biological process) and biological pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For both functional annotation and pathway enrichment, results were used to generate bubble charts ($P < 0.05$) via the OmicShare platform (https://omicshare.com/). The whole framework of network pharmacology is shown in Figure 2.

Composition-Target Molecular Docking

By searching the pubchem website, the SDF structure file of the compound was obtained, and Open Babel 2.3.2 software was used to convert SDF files into PDB files retrieved from the Protein Data Bank (PDB) database (http://www.rcsb.org) in order to map the components of Tan IIA (therapeutic) to interact with the protein targets of CRC (disease) and their disease-relevant targets. The resultant list of predicted targets was inputted into the UniProt (http://www.uniprot.org/) database to obtain current correct corresponding gene names and Uniprot IDs. Duplicates were removed from the list. Finally, therapeutic and disease target lists were compared by means of a Wayne diagram to identify targets shared between the 2 lists.
Bank (http://www.rcsb.org/pdb) database to obtain receptor proteins AKT1 (PDBID: 4EKL), CDK2 (PDBID: 1B39), JUN (PDBID: 5FV8), and TP53 (PDBID: 3Q05). PYMOL 2.3.4 software was used to perform operations such as removing water and ligands on the receptor protein. Furthermore, AutoDock Vina 1.1.2 was used for molecular docking of receptor protein and ligand small molecule. A binding energy of less than 0 indicates that the ligand and the receptor can bind spontaneously. The binding energy of −5.0 kJ/mol was selected as the basis for screening of Tan IIA therapeutic targets for CRC.

Reagents and Equipment

Tan IIA (purity >98 %) was purchased from Dalian Meilun Biotechnology, and dissolved in dimethyl sulfoxide (DMSO). High-glucose Dulbecco’s modified Eagle’s medium (DMEM) and serum were purchased from Gibco (USA), phosphate-buffered saline (PBS), trypsin, penicillin, streptomycin, and double antibody from Hyclone, a cell counting kit-8 (CCK-8) from Boshi de Bioengineering, and propidium iodide from Beijing Dingguo Changsheng Biotechnology.

Cell Culture

Human colorectal cancer cell lines, SW480 and SW620 were purchased from the American Type Culture Collection (ATCC; USA). These cell lines are derived from a single CRC patient, but represent different cancer stages, with SW480 being less far along the continuum of carcinogenesis progression than SW620. Cells were cultured in DMEM containing 10% serum and 1% of each of penicillin and streptomycin, at 37 °C and 5% CO₂ in a saturated humidity incubator.

CCK-8 Detection

Cell proliferation and viability were assessed using CCK-8. Briefly, cell lines were inoculated into a 96-well plate (5000 cells/well for SW480 and 10 000 cells/well for SW620) and incubated at 37 °C in 5% CO₂ until adherence (24 hours). Cells were then treated with Tan IIA at varying concentrations (0, 2.5, 5, 10, 20, 40, and 80 μmol/L) for 48 hours, prior to adding 20 μL CCK-8 solution per well and incubating for 30 minutes in the dark. Finally, per-well absorbance (A) was assessed at a wavelength of 450 nm using Multi-Mode Detection Platform (Molecular Devices Company, SER33270-1235).

Light Microscopy

Cell lines SW480 and SW620 were cultured until they reached the logarithmic growth phase, followed by trypsin digestion-assisted harvesting. Harvested cells were used to inoculate 6-well plates (2 × 10^5 cells/well for SW620 and 8 × 10^4 cells/well for SW480), which were incubated for 24 hours prior to treatment with either vehicle only (DMSO; control group) or vehicle containing Tan IIA at varying concentrations (5 or 10 μmol/L; experimental groups) for 48 hours. Light microscopy (40 × optical
magnification objective) was then used to observe cell morphology.

**Flow Cytometry**

Cells harvested during the logarithmic growth phase were inoculated into 6-well plates (1 × 10⁶ cells/well for SW620 and 5 × 10⁵ SW480 cells/well for SW480), which were incubated for 24 hours prior to treatment with either vehicle only (control group) or vehicle containing Tan IIA (5 mol/L; experimental group) for 48 hours. After addition of 1 ml of propidium iodide (PI) solution to each sample, gentle mixing, and a 30 minutes incubation in the dark, cells were analyzed by flow cytometry (Beckman Cytoflex).

**Results**

**Target Genes Analysis of Tanshinone IIA in Regulating Colorectal Cancer**

Querying the TCMSP database produced 43 Tan IIA (therapeutic) targets. After obtaining their SMILEs, this translated to 172 Tan IIA targets in the SwissTargetPrediction database. After duplicate removal, 152 potential Tan IIA targets remained. Querying the GEO database (GSE44076 and GSE42284), DisGeNET, GeneCards, and TTD databases produced 11371 CRC (disease) targets after de-weighting. The Wayne diagram demonstrates that 128 elements are shared between the therapeutic and disease target lists (Figure 3(A)). The Cytoscape-generated network diagram (Figure 3(B)) includes one component node (therapeutic target) and 126 disease nodes (disease targets).

Highly connected nodes within a network are usually involved in related biological functions. The current PPI network (126 nodes) demonstrated 164 edges (Figure 3(C)), more than would be expected by chance (confidence score >0.95).

**GO Biological Annotation and KEGG Pathway Enrichment Analysis**

Analysis for enrichment of GO terms yielded 157 results, including 104 biological processes (BPs), 26 cellular components (CCs), and 27 molecular functions (MFs). The top 20 BPs (P < 0.05; Figure 4(A)) include “positive regulation of transcription from RNA polymerase II promoter,” “protein processing,” “cell cycle,” and “cell proliferation.” Meanwhile, shared targets were enriched for 84 KEGG pathways. The top 20 pathways (P < 0.05; Figure 4(B)), represented by over 15 genes, include “cancer pathways” (ssc05200, 25 genes), “neuroactive ligand receptor interactions” (ssc04080, 21 genes), “PI3K-Akt pathway” (ssc04151, 18 genes), “RNA in cancer” (ssc05206, 18 genes), “proteoglycans in cancer” (ssc05205, 15 genes), and “prostate cancer” (ssc05215, 15 genes). The top 20 pathways also indicate that Tan IIA likely targets the cell cycle (pathway ssc04110). Results suggest that Tan IIA has a wide range of pharmacological effects in neoplastic disorders,
including regulating tumor cell cycling, proliferation, and apoptosis (including as part of cancer-related pathways).

**Analysis of Docking Results of Tan IIA and CRC Cell Cycle Related Hub Genes**

Based on the biological annotation, Tan IIA functions in regulating cell cycle, thus Tan IIA and hub genes (AKT1, CDK2, JUN, TP53) related with the CRC cell cycle were analyzed by molecular docking. It is generally believed that while the ligand binds to the receptor in a stable conformation, it shows lower energy and the binding of these 2 molecules will happen. The molecular docking results show that the molecular docking affinity of the 4 core genes related to the cell cycle of Tan IIA is less than −5 kJ/mol (please see supplemental file); the conformation of interaction of Tan IIA and indicated genes is shown in Figure 5, which indicates that Tan IIA has good binding activity and could interact with AKT1, CDK2, JUN, and TP53 directly, thereby contributing to regulating the CRC cell cycle and proliferation.

**Effects of Tan IIA on Cell Viability and Proliferation**

The proliferation of SW480 and SW620 cells was inhibited by Tan IIA in a dose-dependent manner (Figure 6(A) and (B)). The EC$_{50}$ of Tan IIA is 40 μmol/L for SW480 cells (with 2.5 μmol/L achieving 50% of the proliferation inhibition achieved by 40 μmol/L) and 20 μmol/L for SW620 cells (with 5 μmol/L achieving 50% of the proliferation inhibition achieved by 20 μmol/L). Therefore, we speculate that the inhibitory effect of Tan IIA on proliferation correlates directly with the degree of CRC progression. The morphologies of SW480 and SW620 cells were only marginally altered by Tan IIA (Figure 6(C)). Consistent with results reported in Section 3.5, both SW480 and SW620 cell lines responded to increasing Tan IIA concentrations by significantly decreasing proliferation. However, morphologies of the majority of cells from both cell lines remained unaltered, although a small subset became smaller and rounder (labelled by arrows in Figure 6(C)), and exhibited impaired adhesive capability.

| Node1  | Node2  | Coexpression | Experimentally determined interaction | Database annotated | Automated textmining | Combined score |
|--------|--------|--------------|--------------------------------------|--------------------|----------------------|----------------|
| PSEN1  | PSEN1  | 0.061        | 0.999                                | 0.9                | 0.954                | 0.999          |
| APH1A  | PSEN2  | 0.079        | 0.997                                | 0.9                | 0.837                | 0.999          |
| NCSTN  | APH1B  | 0.064        | 0.995                                | 0.9                | 0.896                | 0.999          |
| CCNE2  | CDK2   | 0.199        | 0.997                                | 0.9                | 0.856                | 0.999          |
| PSEN1  | APH1A  | 0.087        | 0.999                                | 0.9                | 0.961                | 0.999          |
| FNTA   | FNTB   | 0.087        | 0.983                                | 0.9                | 0.902                | 0.999          |
| RBBP4  | EED    | 0.145        | 0.98                                 | 0.9                | 0.71                 | 0.999          |
| PSEN1  | NCSTN  | 0.083        | 0.999                                | 0.9                | 0.97                 | 0.999          |
| CDK2   | CCNE1  | 0.162        | 0.999                                | 0.9                | 0.928                | 0.999          |
| EZH2   | SUZ12  | 0.113        | 0.985                                | 0.9                | 0.967                | 0.999          |
| TP53   | MDM2   | 0.236        | 0.987                                | 0.9                | 0.974                | 0.999          |
| SUZ12  | EED    | 0.087        | 0.994                                | 0.9                | 0.905                | 0.999          |
| APH1A  | NCSTN  | 0.087        | 0.998                                | 0.9                | 0.979                | 0.999          |
| JUN    | FOS    | 0.656        | 0.879                                | 0.9                | 0.975                | 0.999          |
| EZH2   | EED    | 0.091        | 0.981                                | 0.9                | 0.916                | 0.999          |
| PSEN1  | PSEN2  | 0.061        | 0.998                                | 0.9                | 0.864                | 0.999          |
| EDN1   | EDNRA  | 0           | 0.707                                | 0.9                | 0.974                | 0.999          |
| RBBP4  | SUZ12  | 0.088        | 0.988                                | 0.9                | 0.833                | 0.999          |
| CDKN1A | CCNE1  | 0.063        | 0.994                                | 0.9                | 0.751                | 0.999          |
| APH1A  | PSEN1  | 0.079        | 0.999                                | 0.9                | 0.912                | 0.999          |
| RBBP7  | HDAC1  | 0.521        | 0.944                                | 0.9                | 0.749                | 0.999          |
| CDKN1A | CDK2   | 0.051        | 0.994                                | 0.9                | 0.942                | 0.999          |
| PSEN1  | APH1B  | 0.079        | 0.997                                | 0.9                | 0.795                | 0.999          |
| PSEN2  | NCSTN  | 0.063        | 0.996                                | 0.9                | 0.897                | 0.999          |
| PSEN1  | NCSTN  | 0.061        | 0.96                                 | 0.9                | 0.993                | 0.999          |
| CDKN1A | TP53   | 0           | 0.696                                | 0.9                | 0.97                 | 0.999          |
| PSEN1  | APH1B  | 0.087        | 0.998                                | 0.9                | 0.895                | 0.999          |
| AKT1   | GSK3B  | 0.049        | 0.995                                | 0.9                | 0.918                | 0.999          |
| PSEN2  | APH1B  | 0.079        | 0.997                                | 0.9                | 0.788                | 0.999          |
Effects of Tan IIA on Cell Cycling

Flow cytometry confirmed the network pharmacology prediction that Tan IIA modulates the cell cycle, demonstrating for both cell lines that Tan IIA treatment increases the number of cells (relative to controls) in the G2/M phase (Figure 7), suggesting that Tan IIA inhibits proliferation by preventing cells from progressing to the G1 phase. A more pronounced effect of Tan IIA on cell cycling is noted for SW620 than for SW480 cells, suggesting that cells further progressed along the carcinogenic continuum are more sensitive to such effects of Tan IIA.

Discussion

CRC is a malignant tumor of the digestive tract, with an incidence closely associated with lifestyle factors, including eating habits, physical exercise, obesity, and smoking. Given that CRC typically exhibits rapid cell proliferation, rapid progression to invasiveness, and frequent recurrence, major goals of pharmacological therapeutics may well be described as inhibition of cell cycling and proliferation, as well as induction of apoptosis. One of the most important bioactive constituent of the medicinal plant Salvia miltiorrhiza is Tan IIA, which has been widely used in the treatment of cardiovascular, cerebrovascular, and neoplastic disorders. As mentioned, this compound exhibits multiple anti-neoplastic mechanisms, including inhibiting tumor cell proliferation, invasion, and metastasis, and induction of apoptosis. However, the therapeutic molecular mechanisms of Tan IIA in the context of CRC remain incompletely known. Therefore, we employed empirical validation of network pharmacology-generated hypotheses regarding the possible mechanisms by which Tan IIA opposes CRC, to provide a basis for further research.

Briefly, we identified 125 protein targets common to both Tan IIA and CRC. Certain targets of Tan IIA (e.g., SUZ12, CDK2, Jun, CCNE2, TP53, and AKT1) are known to be important in CRC biology. Cellular functions are executed via the coordinated activities of multiple interacting proteins, and the interactions of these proteins constitute the PPI network. Because altered functioning of key proteins can propagate through the network to cause or relieve disease, these 125 shared targets were used to construct a PPI network. Topology analysis identified a number of highly-connected nodes within the network. For example, TP53 interacts with 4 other proteins (CDK2, Jun, CCNE2, and AKT1), each also exhibiting high degrees of connectedness.

As a tumor suppressor gene, mutation of TP53 within tumor cells will cause expression changes in many p53-regulated genes, resulting in abnormal DNA damage repair, cell cycle arrest, and apoptosis. Generally, tumor suppressor gene inactivation contributes significantly to CRC progression, although TP53 inactivation occurs relatively late during CRC development and is associated with tumor cell aggressiveness. It has been demonstrated that Tan IIA alters the expression of p53-dependent target proteins, thereby inhibiting cell cycling, among other anti-tumor effects. Cyclin-dependent kinase-2 (CDK2) also plays a key role in tumor cell cycling, including progression of the G1-S phase and regulation of the G2 phase. Many studies have shown that CDK2 overexpression is closely associated with the proliferation and apoptosis of tumor cells. Since CDK2 and AKT1 exhibit

Figure 4. GO biological annotation and KEGG pathway enrichment analysis. (A) GO enrichment analysis of 20 hypothetical targets. (B) KEGG pathway enrichment analysis of 20 putative targets.
similar expression trends in most neoplastic disorders, and these trends are involved in promoting tumor cell proliferation while inhibiting tumor cell apoptosis to promote cancer. Identification of CDK2 and AKT1 inhibitors is of great interest in cancer therapeutics. Transcription factor AP-1 (Jun) is able to inhibit expression of the main factors regulating CDK2 (i.e., P21 and CIP1), thereby inhibiting CDK2 activation and thus G1-S progression. Interestingly, Tan IIA is able to maintain high levels of cellular Jun via induction of Nrf2 (a transcription factor). Simultaneously, Tan IIA is able to inhibit signal transduction along the EGFR pathway, thereby decreasing AKT1 activity and inhibiting tumor cell growth and proliferation. By inducing apoptosis, Tan IIA inhibits the proliferation of SW480, SW620, and SW837 cells. Our data suggest that Tan IIA arrests cell cycle in the G2/M phase, and molecular docking results show that Tan IIA could interact with AKT1, CDK2, JUN, and TP53 directly, which might ultimately result in inhibition of cell cycling and proliferation, as well as induction of apoptosis, through altering gene expression via either direct regulation of targets or indirect regulation through modulating the relationships between targets.

In agreement with the above findings, GO term annotation and KEGG pathway enrichment suggest that Tan IIA impacts RNA enzyme transcription, cell proliferation, and the cell cycle (among other BPs), as well as signal pathways, including cancer, PI3K-Akt, HIF-1, and cell cycle pathways, all of which may be
importance in CRC progression. More specifically, enriched GO terms included RNA polymerase II promoter transcription, protein process, G protein-coupled adenosine receptor and endopeptidase activity, cell cycle, proliferation, and apoptosis. This indicates that Tan IIA may act on RNA transcription, protein synthesis, and the activities of various enzymes, in order to modulate cell cycling, proliferation, and apoptosis. Enriched KEGG pathways included predominantly cancer, neuroactive receptor-ligand interaction, colorectal cancer, PI3K-Akt, HIF-1, TNF, and microRNA signaling pathways (although cell cycle and mitosis pathways are also enriched). It has previously been reported that Tan IIA is able to inhibit mitosis, leading to CRC cell apoptosis. Overall, findings suggest that Tan IIA may oppose CRC via multiple targets and pathways, but both GO annotation and KEGG pathway enrichment indicate that cell cycle may play an important role in the therapeutic mechanism of Tan IIA in the context of CRC.

Therefore, in order to validate empirically this network pharmacology prediction, we demonstrated via CCK-8 and flow cytometry, respectively, that in vitro Tan IIA administration inhibits proliferation, as well as arresting the cell cycle in the G2/M phase in 2 hours human CRC cell lines. In summary,
experimental results validate network pharmacology predictions, suggesting that Tan IIA directly regulates CRC cell cycling, thereby inhibiting proliferation and inducing apoptosis. This provides a theoretical basis for further study of the therapeutic mechanisms of Tan IIA in the context of CRC.

Acknowledgments
We would like to thank Editage (www.editage.cn) for English language editing and Miss Mo Bai for figures editing.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: National Natural Science Foundation of China, (grant NO. 81903876). Jilin Province Traditional Chinese Medicine Technology Project (grant No. 2019051).

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Supplemental Material
Supplemental material for this article is available online.

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