Antitumor mechanism research of cryptotanshinone by module-based network analysis

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***Abstract*—Cryptotanshinone (CPT) is one of the major liposoluble ingredients in *Salvia miltiorrhiza* which exerts antitumor activity on several types of cancers. However, the action mechanism of CPT remained to be clarified. The current study aimed to elucidate the antitumor mechanism of CPT based on the protein interaction network (PIN) analysis. A PIN of CPT was constructed with 244 nodes and 778 interactions, and was analyzed by Gene ontology (GO) enrichment analysis based on Markov Cluster algorithm (MCL). Two modules were found to be intimately associated with antitumor. Still further, the antitumor effect of CPT may be partly attributable to inhibiting the activation of the c-Src pathway and overexpression of EGFR, to mediating overexpression of PIAS and activation of EIF2AK3. Therefore, this study may shed new light on the antitumor mechanism and treatment of CPT.**

Keywords—Protein interaction network; module; antitumor actions; cryptotanshinone; GO enrichment analysis

I. INTRODUCTION

Salvia miltiorrhiza (Danshen) is a classical traditional Chinese herbal medicine (TCM) with approximately 1,000 years of clinical application for the treatment of various kinds of diseases [1–4]. In the Dictionary of Traditional Chinese Medicine Prescription, Danshen ranks No.4 among the 1362 classic and empirical prescriptions for cancer therapy by frequency analysis [5]. Therefore, Danshen plays an important role in TCM for cancer treatment. Cryptotanshinone (CPT), a major active component isolated from *Salvia miltiorrhiza*, has been shown to possess various pharmacological activities, such as antitumor, anti-inflammatory and antibacterial [6-8]. Antitumor activity of CPT had become a research focus in recent years. However, the antitumor mechanism of CPT was not fully understood.

Network pharmacology is a novel subject to discover TCM from a systems perspective and at the molecular level [9]. Proteins are vital macromolecules, at both cellular and systemic levels, but they rarely act alone. Protein-protein interactions (PPIs) are major bearers of the biological process. The relevance of PPI as putative therapeutic targets for the development of new treatment is particularly evident in cancer, with several ongoing clinical trials within this area [10].The GO[11] project is a collaborative effort to construct ontologies which facilitate biologically meaningful annotation of gene products. It provides a collection of well-defined biological terms, spanning biological processes, molecular functions and cellular components. GO enrichment is a common statistical method used to identify shared associations between proteins and annotations to GO. Module-network and GO analysis may provide an efficient way to illustrate the molecular mechanism of antitumor action for CPT.

In this study, a network pharmacology approach was applied to analyze the antitumor mechanisms of CPT. PPIs were adopted in constructing a biological network. And the characteristics of scale-free, small-world network and module were analyzed. This paper aimed to further elucidate the antitumor molecular mechanism of CPT, and provide reference for its clinical application and further drug development.

II. MATERIALS AND METHODS

A. Network construction

The targets information of CPT was extracted from ChEMBL (https://www.ebi.ac.uk/chembl/#) and STITCH 3.1 (http://stitch.embl.de/). ChEMBL is an open large-scale bioactivity database, with the information largely manually extracted from the medicinal chemistry literatures [12]. STITCH [13] is a database for protein–chemical interactions that integrates many sources of experimental and manually curated evidence with text-mining information and interaction predictions. Every protein-chemical interaction has a confidence score.

The PPIs information was obtained from the online update databases of String 9.1(http://string-db.org) which provides uniquely comprehensive coverage and ease of access to both experimental as well as predicted PPI information. Thereafter, the known and predicted associations are scored and integrated [14].

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B. Network analysis

The topological properties of the PPI network, such as degree distribution, clustering coefficient and average shortest path were analyzed by Network Analyzer [15] in Cytoscape software. Degree distribution refers to the number of connections between proteins of the network; Clustering

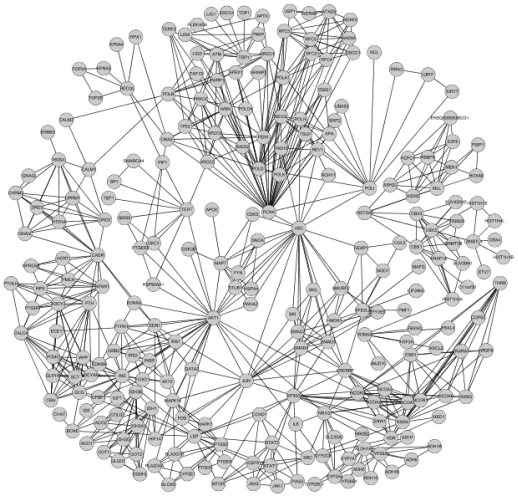

Fig. 1. Protein interaction network of CPT. The nodes and edges indicated the proteins and their relations

coefficient is the average density of the node neighborhoods; Average shortest path means the average density of shortest paths between all pairs of nodes [16]. Properties of scale-free, small word and modularity of the PIN were also investigated based on the topological parameters.

Functional modules of the network were explored by the MCL [17]. MCL simulated a flow on the graph by calculating successive powers of the associated adjacency matrix and the value of the inflation parameter strongly influenced the number of clusters, which was superior to other algorithms, e.g. RNSC, MCODE and SPC with highlighting the robustness to graph alterations [18]. Based on the identified modules, GO enrichment analysis was utilized to predict possible biological roles of the modules by evaluating the involved biological processes, using the BinGO [19] plugin for Cytoscape.

III. RESULTS AND DISCUSSION

A. Construction of the network

6 and 30 human proteins were extracted from STITCH 3.1 and ChEMBL (up to Junuary of 2014), respectively. 35 human proteins were obtained as CPT targets after removing a repeat protein. Research had shown that the binding affinity (IC_{50}) of CPT and STAT3 was 4600nm, however, the IC_{50} are not available because CPT can inhibit or activate other proteins [20, 21]. The targets were listed in Table 1. PPIs information of targets whose confidence score was higher than 0.7 was imported in Cytoscape 2.8.3 [22], then carried union calculation and removed duplicated edges of PPIs using Advanced Network Merge [23] of Plugins, lastly, selected the largest connected subgraph as the PIN of CPT which included 244 nodes and 778 edges. The PIN of CPT was shown in figure 1.

B. Network analysis

1) Topological analysis

TABLE I. THE LIST OF TARGETS OF CPT.

Targets	UniProt ID	Source	Targets	UniProt ID	Source
ALDH1A1	P00352	ChEMBL	POLH	Q9Y253	ChEMBL
ATAD5	Q96QE3	ChEMBL	POLI	Q9UNA4	ChEMBL
BAZ2B	Q9UIF8	ChEMBL	POLK	Q9UBT6	ChEMBL
CBX1	P83916	ChEMBL	RECQL	P46063	ChEMBL
FEN1	P39748	ChEMBL	RGS4	P49798	ChEMBL
GLP1R	P43220	ChEMBL	SMAD3	P84022	ChEMBL
GMNN	O75496	ChEMBL	STAT3	P40763	BOTH
IDH1	O75874	ChEMBL	TDP1	Q9NUW8	ChEMBL
KAT2A	Q92830	ChEMBL	TERT	O14746	ChEMBL
KDM4A	O75164	ChEMBL	THRB	P10828	ChEMBL
KDM4E	B2RXH2	ChEMBL	VDR	P11473	ChEMBL
L3MBTL1	Q9Y468	ChEMBL	WRN	Q14191	ChEMBL
MAPT	P10636	ChEMBL	BCHE	P06276	STITCH
MBNL1	Q9NR56	ChEMBL	INS	P01308	STITCH
MLL	Q03164	ChEMBL	EDN1	P05305	STITCH
NFE2L2	Q16236	ChEMBL	PTGS2	P35354	STITCH
NPSR1	Q6W5P4	ChEMBL	NR1I2	O75469	STITCH
POLB	P06746	ChEMBL			

All the topological parameters were calculated and shown in Table 2.

As shown in Figure 2A, the degree distribution of the PIN of CPT followed the power law distribution and the equation is y=85.694x-1.181. So the PIN of CPT was a scale-free network that possessed fragility and robustness [24-26].

As shown in Figure 2B, network path length was mostly concentrated in 3-5 steps. The shortest path length between any two proteins of 4.958 links was calculated. Small world networks have a property that Characteristic path length is small [27]. This indicated that most proteins were closely

TABLE II. THE SIMPLE PARAMETERS OF PROTEIN INTERACTION NETWORK OF CPT

Parameters	PIN of CPT
Clustering coefficient	0.659
Connected components[a]	1
Network diameter[b]	12
Network centralization[c]	0.123
Shortest path	59292(100%)
Characteristic path length	4.958

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Network heterogeneity[d]	0.743

[a.] The connected component is 1 that indicates the network has no other subgraph.

[b.] The network diameter is the greatest distance between any pair of vertices

[c.] Network centralization is a network index that measures the degree of dispersion of all node centrality scores in a network

[d.] network heterogeneity can characterize the degree of uneven distribution of the network

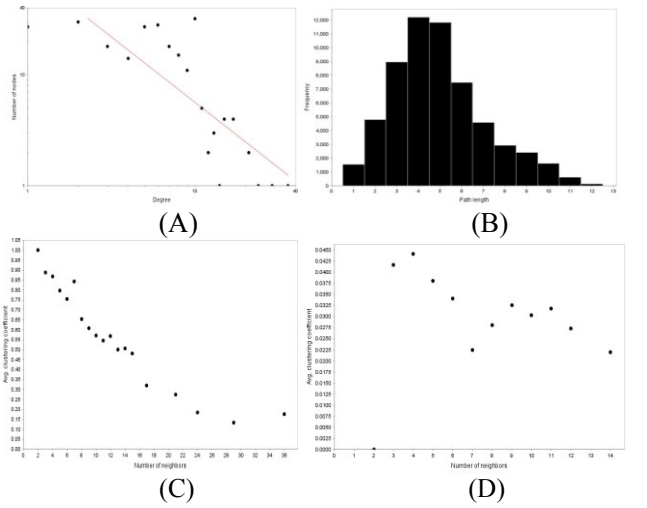

Fig. 2. Topological properties of network**. (A)** The degree distribution of CPT network; (**B)** Shortest path length distribution of CPT network; (**C**) Average clustering coefficient of CPT network; (**D**) Average clustering coefficient of random network

linked and the PIN of CPT was a small world network.

As shown in Figure 2 (C and D), compared with random network whose number of nodes and edges were identical to the PIN of CPT, the clustering coefficient of PIN was higher. In graph theory, clustering coefficient is the measure of the clustering degree of nodes. It indicated the PIN of CPT was more modularity. These results suggested that the network exhibited scale-free property, small world property and modular architecture.

2) Clustering and GO enrichment analysis

22 modules were identified with MCL algorithm (shown in Figure 3). All 22 modules included 236 of the total 244 proteins.

The results of functional enrichment analysis using BinGO are shown in Table 3, showed that CPT played a pharmacodynamics with the biological processes, such as DNA metabolic process, tricarboxylic acid metabolic process, icosanoid metabolic process, antitumor response, etc. And module 8 and 13 are related to antitumor action.

Module 8 contained proteins such as STAT3, JAK1, JAK2, PIAS3, SRC, EGFR. As a member of STAT family which transduces extracellular signals and regulates transcription of target genes, STAT3 has been proved to be the most intimately linked to tumorigenesis [28]. Dae-Seop Shin had studied that CPT exerted antitumor property by inhibiting STAT3 [20]. STAT3 is activated by JAK [21] and dysregulation of the pathway is frequently observed in primary tumors [29].It was previously reported that CPT induced inhibition of breast tumor growth through the JAK/STAT signaling pathway [30]. PIAS proteins were important transcriptional co-regulators of the JAK/STAT growth when overexpressed [31]. CPT may possess antitumor activity associating with overexpression of PIAS. SRC and STAT3 are coordinately altered in many human signaling pathway and PIAS inhibited human lung cancer cell tumors [32-34]. The activation of the c-Src pathway leading to the promotion of survival, angiogenesis, proliferation and invasion pathways has been observed in about 50% of tumors [35]. SRC was activated by EGFR [36] while EGFR overexpression or overactivition had been associated with a number of cancers, including lung cancer, anal cancers, which produced uncontrolled cell division [37]. The analysis of module 8 indicated that the antitumor effects of CPT may be attributed to inhibiting the activation of the c-Src pathway and overexpression of EGFR.

Antitumor activity of Module 13 was closely related to regulation of apoptotic process including HMOX1, EIF2AK3, etc. HMOX1 belongs to the heme oxygenase family, which was actived at high concentrations of heme and was thought to function as an oxidative stress indicator [38]. It was reported that HMOX1 was involved in estrogen-induced cell apoptosis progress. Antitumor effects of CPT may be relevant to HMOX1. EIF2AK3, also known as PERK, is a type I membrane protein located in the endoplasmic reticulum (ER) where it was induced by ER stress [39]. However, activation of PERK resulted in phosphorylation of eukaryotic translation initiation factor 2 subunit α (eIF2α), which lead to

TABLE III. GO BIOLOGICAL PROCESS TERMS OF THE MODULES DISPLAY PARTIALLY

Modules	GO terms	P-value
Module 1	cellular response to endogenous stimulus	7.28E-36
Module 2	response to DNA damage stimulus	1.89E-39
Module 3	transcription initiation from RNA polymerase II promoter	4.95E-25
Module 4	xenobiotic metabolic process	8.62E-21
Module 5	histone lysine methylation	1.41E-12
Module 6	tricarboxylic acid cycle	1.57E-18
Module 7	chromatin organization	2.84E-10
Module 8	JAK-STAT cascade	1.71E-09
Module 9	telomere maintenance	6.97E-12
Module 10	icosanoid biosynthetic process	1.74E-15
Module 11	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	5.42E-13
Module 12	double-strand break repair	1.10E-10
Module 13	regulation of apoptotic process	3.59E-05
Module 14	transcription initiation from RNA polymerase I promoter	1.25E-05
Module 15	mitotic recombination	3.52E-11
Module 16	chromatin organization	2.04E-04
Module 17	chromatin organization	3.07E-08
Module 18	neuropeptide signaling pathway	1.03E-06

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Module 19	neural crest cell development	2.44E-10
Module 20	synaptic transmission, cholinergic	1.36E-06
Module 21	steroid metabolic process	1.11E-06
Module 22	regulation of cell communication by electrical coupling involved in cardiac conduction	1.94E-08

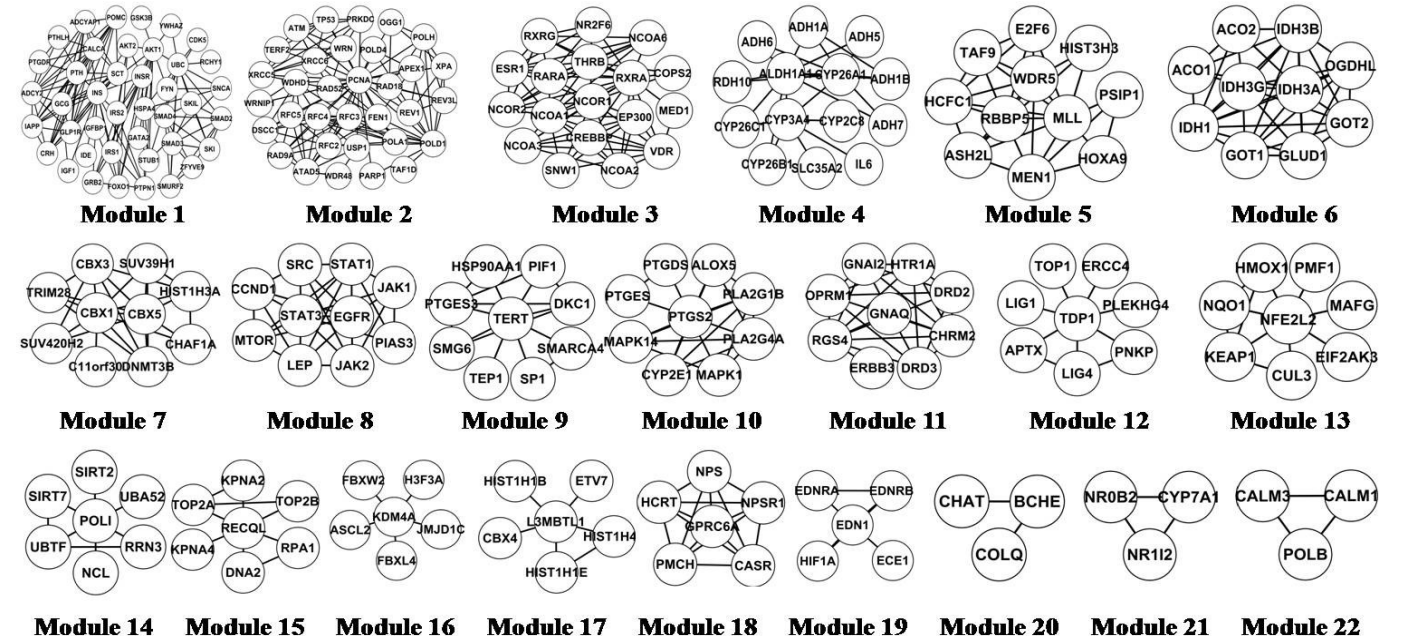

Fig. 3. Modules in the PIN of CPT. With the MCL algorithm, 22 modules are extracted from the netwrok.

suppression of general protein translation [40]. Kinnosuke Yahiro showed that EIF2AK3 could induce cell apoptosis via the ubiquitin-proteasome system [40]. It had been reported that CPT induced ER stress-mediated apoptosis [41]. This indicated that CPT may exert antitumor properties though activation of EIF2AK3.

C. Conclusion

In this paper, the PIN of CPT exhibited the properties of scale-free property, small world property and modular architecture based on analysis of topological parameters. A module-based network analysis approach was proposed to expound the anti-inflammatory mechanism of CPT. The antitumor effects of CPT may be partly attributed to inhibiting the activation of the c-Src pathway and overexpression of EGFR, to mediating overexpression of PIAS and activation of EIF2AK3. Further experiments are needed to confirm the conclusions.

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