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Anti-inflammatory mechanism research of tanshinonellA by module-based network analysis[1]

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Abstract. Tanshinone IIA (Tan IIA) is one of the major fat-soluble ingredients in *Salvia miltiorrhiza* which has been widely used for various inflammatory conditions associated with cardiovascular and cerebrovascular disorders. However, the underlying anti-inflammatory mechanisms of Tan IIA are incompletely understood. The purpose of this study was to illuminate the anti-inflammatory mechanism of Tan IIA based on the protein interaction network (PIN) analysis. A PIN of Tan IIA was constructed with 281 nodes and 814 interactions and analyzed by gene ontology (GO) enrichment analysis based on Markov Cluster algorithm (MCL). Three modules were associated with anti-inflammatory actions. The most interesting finding of this study was that the anti-inflammatory effect of Tan IIA may be partly attributable to the mediate activation of TRAF2, TRAF3 and TRAF6, to inhibit the toll-like receptor signaling pathway and combine with AGER. Therefore, the module-based network analysis approach will be a new method for better understanding the anti-inflammatory mechanism of Tan IIA.

Keywords: Protein interaction network, module, anti-inflammatory actions, Tanshinone IIA, GO enrichment analysis

1. Introduction

Radix Salvia Miltiorrhiza (Danshen) is widely used in traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases, such as angina pectoris, hyperlipidemia, and acute ischemic stroke [1–3]. Tan IIA is one of the main fat-soluble ingredients of *Salvia miltiorrhiza* which exerts anti-inflammatory actions in many experimental disease models [4,5], so Tan IIA has been widely used for various inflammatory conditions associated with cardiovascular and cerebrovascular disorders [6]. Tan IIA could reduce LPS-induced pro-inflammatory cytokine (IL-1β, IL-6, and TNF-α) released from macrophages during inflammation [7–9], however, the underlying anti-inflammatory mechanisms of Tan IIA are incompletely understood. In the current medical world, it is popular to explain the pathogenesis of cardiovascular disease from the perspective of

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inflammation, and then to intervene in all aspects of inflammation, in order to develop new drugs or new therapies for cardiovascular diseases. Based on gene ontology (GO) analysis and module-network, this paper systematically illuminated anti-inflammatory mechanism of Tan IIA and elaborately illustrated the treatment of Tan IIA for cardiovascular and cerebrovascular diseases.

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. Therefore, several properties of PPI such as allosteric sites and hotspots, have been incorporated into drug-design strategies [10,11]. The GO [12] project is a collaborative effort to construct ontologies so as to 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.

In this study, a network pharmacology approach was applied to analyze the anti-inflammatory mechanisms of Tan IIA. PPIs were adopted in constructing a biological network. And scale-free, small-world network and module characteristics were analyzed. This paper aimed to provide a new approach to study anti-inflammatory mechanisms of Tan IIA systematically.

2. Materials and methods

2.1. Network construction

The target's information of Tan IIA was extracted from ChEMBL (https://www.ebi.ac.uk/chembl/#) and STITCH3.1 (http://stitch.embl.de/). ChEMBL is a database of bioactive drug-like small molecules and has grown to become "the most comprehensive ever seen in a public database" because of its coverage of available bioactivity data [13,14]. STITCH [15] is a database of protein-chemical interactions combining repository of data that captures as much as possible the publicly available knowledge on protein-chemical associations.

The PPI information was obtained from the online updated databases of String 9.1 (http://string-db.org). It provides uniquely comprehensive coverage and ease of access to both experimental as well as predicted PPI information. Interactions in STRING are provided with a confidence score, and accessory information such as protein domains and 3D structures is also available, all within a stable and consistent identifier space.

2.2. Network analysis

The characterization of biological networks by means of graph-topological properties has become very popular for gaining insight into the organization and structure of the resultant large complex networks [16–20]. Therefore, a plugin for Cytoscape Network Analyzer [21] was employed to compute and display a comprehensive set of topological parameters, including connected components, network diameter, radius, density, centralization, heterogeneity, clustering coefficient, characteristic path length, and the distributions of node degrees, and so on. Properties of scale-free, small word and modularity of the PIN were also investigated based on the topological parameters.

The MCL [22,23] was applied to identify functional modules in the PIN, which simulates a flow on the graph by calculating successive powers of the associated adjacency matrix, and the value of the inflation parameter strongly influences the number of clusters. MCL [24] with highlighting the

robustness to graph alterations is superior to other algorithms, e.g. RNSC [25], MCODE [26] and SPC [27]. 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 [28] plugin for Cytoscape.

3. Results and discussion

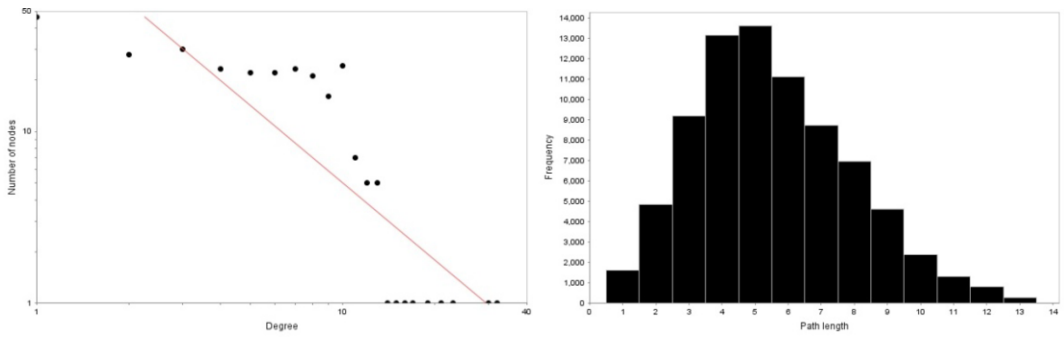
3.1. Construction of the network

Using "Tanshinone IIA" as the key word, 9 and 35 human proteins were respectively extracted as Tan IIA from STITCH 3.1 and ChEMBL (gained the data on December of 2013); the targets and their binding affinities were listed in Table 1. PPI information of the targets with their confidence score above 0.7 was imported in Cytoscape 2.8.3 [31], then union calculation was carried out, followed by the removal of duplicated edges of PPIs using Advanced Network Merge [32] of Plugins, and the largest connected subgraph was selected as the PIN of Tan IIA. Due to the limitation of the current study, there are still unclear human protein interactions, thus the network constructed was not fully connected, and the largest network was connected with subgraph research. The generated network contained 281 nodes and 814 edges which indicated the proteins and their relations.

3.2. Network analysis

3.2.1. Topological analysis

Biological networks have been proposed to have scale-free topology whose degree distribution follows a power law distribution $P(k) \sim k^{-\gamma}(\gamma<3)$ [33]. Scale-free networks possess fragility and robustness [34–36] which allow networks to have a fault tolerant behavior. As shown in Figure 1A, interaction degree distribution exhibited power law behavior and the PIN of Tan IIA was a scale-free network.



(A) (B)

Fig. 1. The degree distribution and the shortest path distribution of the PIN of Tan IIA. (A) The degree distribution of the PIN of Tan IIA: the network followed the power law distribution, the equation is $y=155.27x^{-1.491}$, so the PIN of Tan IIA was scale-free; (B) Distribution of the shortest path between pairs of proteins in the Tan IIA network: On average, any two proteins in the network were connected via 5.547 links.

Table 1

The list of targets and their binding affinities of Tan IIA

Targets	UniProt ID	IC50(nM)	Source	Targets	UniProt ID	IC50(nM)	Source
AKR1B1	P15121	8690	ChEMBL	NFE2L2	Q16236	-	ChEMBL
ALDH1A1	P00352	-	ChEMBL	POLB	P06746	-	ChEMBL
ALOX12	P18054	-	ChEMBL	POLH	Q9Y253	-	ChEMBL
APAF1	O14727	4820	ChEMBL	POLI	Q9UNA4	-	ChEMBL
ATAD5	Q96QE3	-	ChEMBL	POLK	Q9UBT6	-	ChEMBL
BAZ2B	Q9UIF8	-	ChEMBL	RECQL	P46063	-	ChEMBL
CBFB	Q13951	-	ChEMBL	RGS4	P49798	-	ChEMBL
CBX1	P83916	-	ChEMBL	RUNX1	Q01196	-	ChEMBL
EHMT2	Q96KQ7	-	ChEMBL	SMAD3	P84022	-	ChEMBL
FEN1	P39748	-	ChEMBL	TDP1	Q9NUW8	-	ChEMBL
HPGD	P15428	-	ChEMBL	TP53	P04637	-	ChEMBL
HSD17B10	Q99714	-	ChEMBL	USP1	O94782	-	ChEMBL
KAT2A	Q92830	-	ChEMBL	VDR	P11473	-	ChEMBL
KDM4A	O75164	-	ChEMBL	CD40*	P25942	-	STITCH
KDM4DL	B2RXH2	-	ChEMBL	CYP1A1*	P04798	-	STITCH
L3MBTL1	Q9Y468	-	ChEMBL	CYP3A4*	P08684	-	STITCH
LMNA	P02545	-	ChEMBL	FSD1*	Q9BTV5	-	STITCH
MAPK1	P28482	-	ChEMBL	HMGB1*	P09429	-	STITCH
MAPT	P10636	-	ChEMBL	KCNE1*	P15382	-	STITCH
MBNL1	Q9NR56	-	ChEMBL	NFKB1	P19838	-	STITCH
MEN1	O00255	-	ChEMBL	SPG7	Q9UQ90	-	STITCH
MLL	Q03164	-	ChEMBL	TNF	P01375	-	STITCH

Notes: Tan IIA can inhibit or activate other proteins [29,30], so the IC50 are not available. Confidence score of the targets from STITTCH is above 0.7. *Targets of Tan IIA were extracted from both ChEMBL and STITCH.

Small world networks have a property that characteristic path length is short [37].The shortest path length between any two proteins of 5.547 links was calculated. As shown in Figure 1B, network path length was mostly concentrated in 4-6 steps, which meant that most proteins were closely linked and the PIN of Tan IIA was a small world network.

In graph theory, a clustering coefficient is a measure of the degree to which nodes in a graph tend to cluster together. Compared with random network whose number of nodes and edges are the same as PIN of Tan IIA, the clustering coefficient of PIN was higher and thus the PIN of Tan IIA was more modular. These results suggested that the network exhibited scale-free property, small world property and modular architecture. All topological parameters were shown in Table 2.

Table 2

The simple parameters of protein interaction network of Tan IIA and random network

networks / Parameters	PIN of Tan IIA	Random Network
Clustering coefficient	0.610	0.021
Connected components	1	2
Network diameter	13	6
Network radius	7	4
Network centralization	0.094	0.022
Shortest path	78680(100%)	78120(100%)
Characteristic path length	5.547	3.376
Network density	0.021	0.021
Network heterogeneity	0.764	0.402

Notes: The connected component is 1 that indicates the network has no other subgraph. The network diameter is the longest distance between any pair of vertices and the radius of a graph is the minimum eccentricity of any vertex. Network centralization is a network index that measures the degree of dispersion of all node centrality scores in a network. And network heterogeneity can characterize the degree of uneven distribution of the network.

3.2.2. Clustering and GO enrichment analysis

With the MCL algorithm, 21 modules were identified (shown in Figure 2). All 21 modules included 799 of the total 814 proteins.

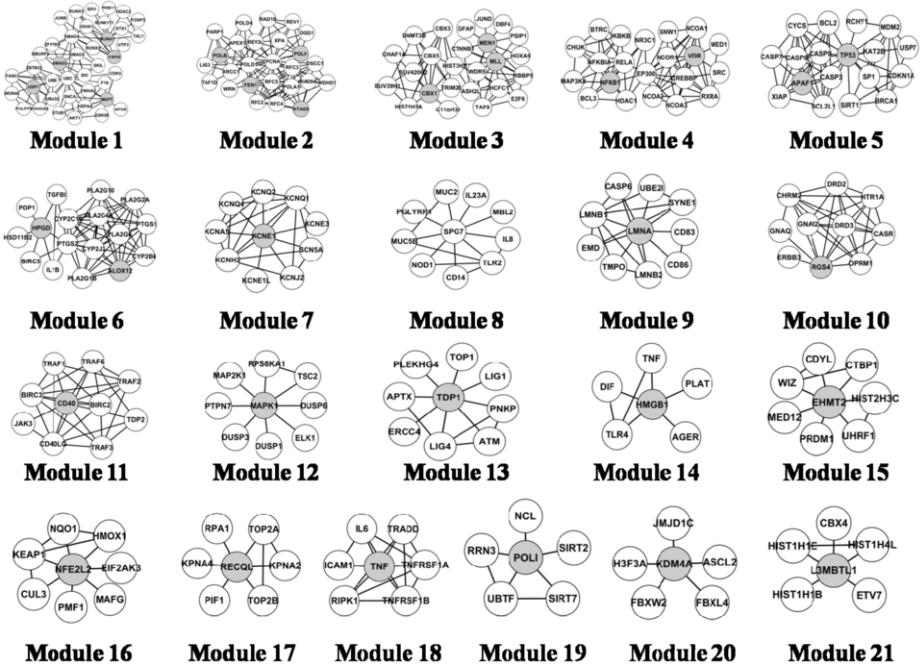


Fig. 2. Modules in the PIN of Tan IIA. With the MCL algorithm, 21 modules are extracted from the network. The gray nodes present seed nodes and the others are nodes that interact with seed nodes.

Functional enrichment analysis has been shown to be conducive to gaining insight into the shared underlying biological function of the proteins associated with a module [38]. Functional enrichment was carried out using BinGO. For each module, the most significant GO biological processes were assigned. The results are shown in Table 3.

The results of functional enrichment analysis showed that Tan IIA played an important role in pharmacodynamics with the biological processes, such as positive regulation of cellular process, DNA metabolic process, nucleic acid metabolic process, apoptotic signaling pathway, inflammatory response and so on. And modules 11, 12 and 14 are related to the regulation of inflammatory process.

Table 3

GO biological process terms of the modules display partially

Modules	GO terms	P-value
Module 1	cellular response to growth factor stimulus	1.07E-22
Module 2	DNA repair	5.13E-32
Module 3	chromatin organization	9.20E-18
Module 4	intracellular receptor mediated signaling pathway	3.12E-22
Module 5	apoptotic signaling pathway	1.77E-20
Module 6	icosanoid metabolic process	1.73E-16
Module 7	potassium ion transport	1.39E-15
Module 8	response to bacterium	4.57E-11
Module 9	cellular component disassembly involved in execution phase of apoptosis	2.23E-06
Module 10	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	1.35E-12
Module 11	NIK/NF-kappaB cascade	3.00E-08
	positive regulation of I-kappaB kinase/NF-kappaB cascade	1.13E-07
Module 12	TRIF-dependent toll-like receptor signaling pathway	1.52E-12
	toll-like receptor 2 signaling pathway	1.52E-12
Module 13	double-strand break repair	7.40E-13
Module 14	inflammatory response	4.47E-03
	positive regulation of NF-kappaB transcription factor activity	4.65E-03
Module 15	chromatin organization	7.86E-06
Module 16	proteasomal ubiquitin-independent protein catabolic process	1.81E-07
Module 17	mitotic recombination	3.69E-08
Module 18	cytokine-mediated signaling pathway	3.71E-11
Module 19	transcription initiation from RNA polymerase I promoter	8.93E-06
Module 20	chromatin organization	2.04E-04
Module 21	chromatin organization	3.07E-08

Notes: P-value is the probability of obtaining the observed effect, a very small P-value indicates that the observed effect is very unlikely to have arisen purely by chance, and therefore provides evidence against the null hypothesis.

Module 11 contains proteins such as CD40, TRAF2, TRAF3 and TRAF6. CD40 is a member of the TNF-receptor superfamily which has been found to play an important role in mediating a broad variety of immune and inflammatory responses [39]. It had been reported that Tan IIA exerted anti-inflammatory properties by reducing levels of CD40 [29,30]. TRAF2 [40–42], TRAF3 [41,43] and TRAF6 [41,43] had been shown to interact with CD40 and demonstrated to mediate activation of NF-κB/JNK/p38. TRAF2 mediated TNF-induced NF-κB activation and impaired JNK activation [44,45]. Overexpression of TRAF3 inhibited CD40 mediating antibody secretion [46] and had an inhibitory effect on NF-κB activation through LT-βR [47]. TRAF6 inhibited NF-κB activation by IL-1 rather than TNF and activated JNK and p38 when overexpressed [48,49]. At the same time, anti-inflammatory effects of Tan IIA may be attributed to its modulation of NF-kappaB, JNK and p38 pathway [50–54], then Tan IIA may possess anti-inflammatory activity by TRAF2, TRAF3 and TRAF6.

Module 12 was closely related to the Toll-like receptor family(TLRs), which are synergistic in mediating production of cytokines such as interleukin 12 and tumor necrosis factor α(TNF-α) [55]. Previous study had demonstrated that Tan IIA can reduce the level of the pro-inflammatory cytokines TNF-α to resist inflammatory [56–59]. This indicates that Tan IIA may exert anti-inflammatory properties through the inhibition of toll-like receptor signaling pathway.

Module14 possesses anti-inflammatory activity including HMGB1, TLR4 and RAGE. HMGB1 is a cytokinemediator of inflammation [60], and its mechanism of inflammation and damage is binding to TLR4, which mediates HMGB1-dependent activation of macrophage cytokine release [61,62]. AGER, which considered as a receptor for HMGB1, is involved in inflammation resolution leading to tissue repair or alternatively in its perpetuation leading to chronic inflammation [63,64]. It had been reported that the anti-inflammatory effects of Tan IIA were relevant to attenuation of systemic accumulation of HMGB1 [65]. This suggests that AGER might be a potential target of Tan IIA to treat inflammation.

4. Conclusion

In this paper, the PIN of Tan IIA exhibits scale-free property, small world property and modular architecture based on the analysis of topological parameters. A module-based network analysis approach was proposed to expound the anti-inflammatory mechanism of Tan IIA. The anti-inflammatory effects of Tan IIA may be partly attributable to mediate activation of TRAF2, TRAF3 and TRAF6, to inhibit the toll-like receptor signaling pathway and combine with AGER. This indicates that the module-based network analysis approach can serve as a new approach to expound the anti-inflammatory mechanism of Tan IIA. However, further experiments are needed to confirm the conclusions.

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