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The anticancer mechanism investigation of Tanshinone II$_A$ by pharmacological clustering in protein network

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Abstract

Background: Cancer is the second most common cause of death globally. The anticancer effects of Tanshinone II$_A$ (Tan II$_A$) has been confirmed by numerous researches. However, the underlying mechanism remained to be integrated in systematic format. Systems biology embraced the complexity of cancer; therefore, a system study approach was proposed in the present study to explore the anticancer mechanism of Tan II$_A$ based on network pharmacology.

Method: Agilent Literature Search (ALS), a text-mining tool, was used to pull protein targets of Tan II$_A$. Then, pharmacological clustering was applied to classify obtained hits, the anticancer module was analysed further. The top ten essential nodes in the anticancer module were obtained by ClusterONE. Functional units in the anticancer module were catalogued and validated by Gene Ontology (GO) analysis. Meanwhile, KEGG and Cell Signalling Technology Pathway were employed to provide pathway data for potential anticancer pathways construction. Finally, the pathways were plotted using Cytoscape 3.5.1. Furthermore, in vitro experiments with five carcinoma cell lines were conducted.

Results: A total of 258 proteins regulated by Tan II$_A$ were identified through ALS and were visualized by protein network. Pharmacological clustering further sorted 68 proteins that intimately involved in cancer pathogenesis based on Gene Ontology. Subsequently, pathways on anticancer effect of Tan II$_A$ were delineated. Five functional units were clarified according to literature: including regulation on apoptosis, proliferation, sustained angiogenesis, autophagic cell death, and cell cycle. The GO analysis confirmed the classification was statistically significant. The inhibiting influence of Tan II$_A$ on p70 S6K/mTOR pathway was revealed for the first time. The in vitro experiments displayed the selectivity of Tan II$_A$ on HeLa, MDA-MB-231, HepG2, A549, and ACHN cell lines, the IC$_{50}$ values were 0.54 μM, 4.63 μM, 1.42 μM, 17.30 μM and 204.00 μM, respectively. This result further reinforced the anticancer effect of Tan II$_A$ treatment.

Conclusions: The current study provides a systematic methodology for discovering the coordination of the anticancer pathways regulated by Tan II$_A$ via protein network. And it also offers a valuable guidance for systematic study on the therapeutic values of other herbs and their active compounds.

Keywords: Tanshinone II$_A$, Antineoplastic agents, Network pharmacology, Pharmacological clustering

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Background

Tanshinone II$_A$ (Tan II$_A$) is a phenanthrene-quinone extracted from a medicinal herb Danshen, *Salvia miltiorrhiza* Bunge. The herb has been used in Traditional Chinese medicine (TCM) practice for over a millennium. It possesses efficacy of promoting blood circulation and preventing blood stagnation based on years of clinical observation. Chinese medicine practitioners have applied herbs with such function to treat cancerous diseases based on detectable clinical assessments [1–3] even before the modern concept of cancer was defined.

Nowadays, cancer is the second most common cause of death in developed and several developing countries. The conventional therapies, radiotherapy and chemotherapy, have showed their limitations and drawbacks, for instance, severe side-effects, intolerance and increasing resistance. Hence the need for developing a series of new anticancer drugs is growing. Phytochemical compounds are a promising source, as groups of plant derivatives, such as paclitaxel, trabectedin, and vincristine, have already been developed into therapeutic drugs. The urgent need for novel anti-tumour agents hastens the researches on anticancer activity of Danshen and its major active component. Tan II$_A$ has been considered to possess similar function as its parent herb. In modern medicinal studies, it has been reported to exhibit a variety of pharmacological activities, including but not limited to anti-inflammatory, anti-oxidative, anti-atherosclerosis, and anticancer [4, 5]. The anticancer activity of Tan II$_A$ has attracted numerous interests in the past decade. The anticancer effects and potential mechanisms of Tan II$_A$ have been studied extensively in various cancer cell lines and showed promising activity via in vivo experiments [6, 7] and clinical studies [8].

The large number of researches on anticancer effect of Tan II$_A$ provided a great opportunity for effective data mining and network pharmacological study on this subject. Network pharmacology, based on network biology and systems biology, has been favoured by TCM researchers. The network pharmacology shares same guiding theory with TCM practice which is 'parts can be understood only in its relation to the whole [9]'. In the present case, the regulatory effects of Tan II$_A$ on its distinctive targets can provide detectable therapeutic outcomes only when they are added-up. TCM network pharmacology integrates TCM theory with molecular networks that focuses on the systematic effects of drug targets on the biological network [10]. It offers a novel researching approach for mapping the anticancer mechanisms of Tan II$_A$ and identifying potential protein targets that coordinated to produce synergetic effect.

With the exponential expansion of publications, literature mining became a critical tool for a labour-saving secondary study based on the primary experiment data. Text mining by a Cytoscape plug-in, Agilent Literature Search (ALS), retrieved results from various reliable data resources such as PubMed and provided evidence-based outcomes for further manual inspection. This approach was similar to the 'lightweight' approach to text mining which proved robust and sustainable [11]. KEGG has been characterized as a third-generation approach [12] in pathway analysis. It offers valuable information on the interaction of proteins for the present system study. Pharmacological clustering, on top of topological analysis has been applied in other studies, was utilized in the present network study. This clustering method was developed based on National Center for Biotechnology Information (NCBI) oncology database, it generated modules based on the function of individual protein instead of topological features of the network. The modules derived from pharmacological clustering were intimately connected to the function, the proteins in the same module were more likely to produce similar pharmacological effects in response to a given chemical entity.

In addition, to evaluate the potential of regulatory effects of Tan II$_A$ on various cancer types, the inhibitory effects were tested by in vitro cell proliferation experiments with several cell lines. Breast cancer, lung cancer, kidney cancer and cervical cancer were among the most common cancers [13, 14], hence the sensitivity and specificity of these cancers to Tan II$_A$ treatment was assessed on the representative cell lines of these cancers.

The present work aimed to understand the antineoplastic action of Tan II$_A$ in a systematic and comprehensive angle. Therefore, network pharmacological approaches combing in vitro experiments were applied to construct a reliable systematic network to illustrate the mechanism and would also valuable for further research on the therapeutic values of Chinese herbal products.
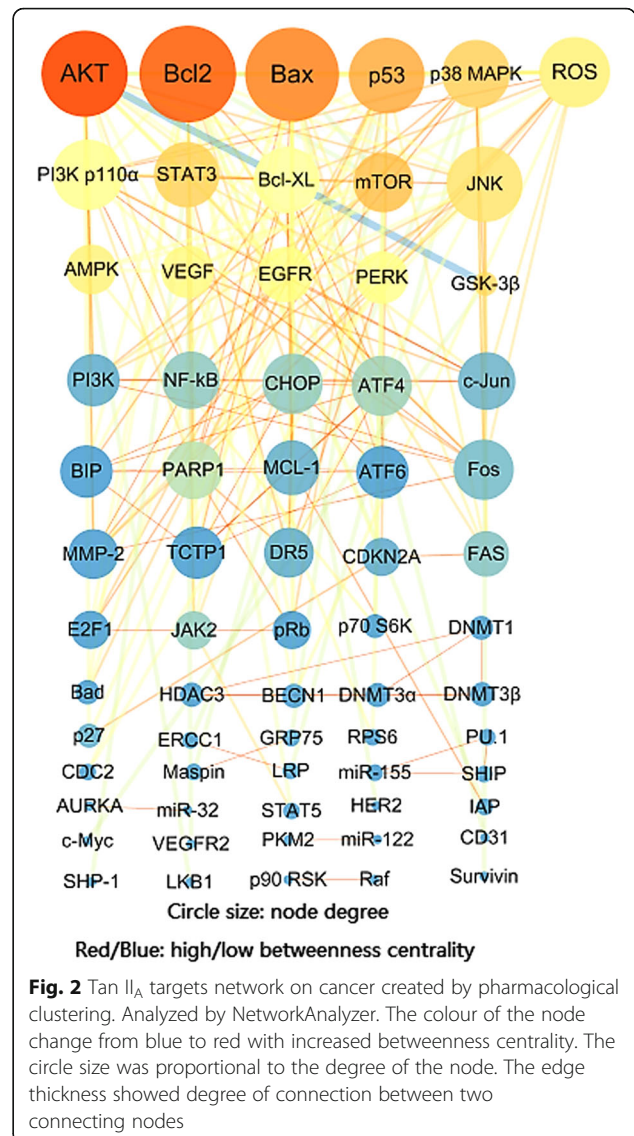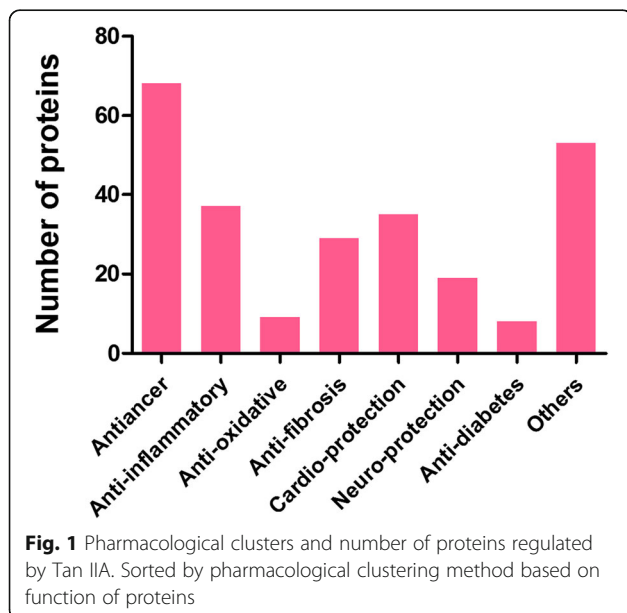
Results

A network of all proteins regulated by Tan II$_A$

The network constructed by ALS data mining contained all proteins under the influence of Tan II$_A$ treatment. Target proteins were identified by official symbol and other aliases, the abbreviations used in research papers could accidentally coincide with them. Therefore, checks against supporting evidences for each hit have been done to ensure correct protein identification, false positives were processed. For example, 1. aliases: search hit "pkb" and "akt1" both stand for "AKT serine/threonine kinase 1", "jun" and "mapk8" for "c-Jun N-terminal kinases" they were merged as node "Akt" and "JNK" respectively; 2. false identification, "FBS" (Fetal bovine serum) in original research paper [15] was falsely identified as "F-box protein 8", the node was subsequently deleted. In total,

396 nodes were retrieved, among which 258 proteins were regulated by Tan II$_A$. The functions of proteins were obtained from NCBI databases and other recent researches. The pharmacological activities of Tan II$_A$ could be classified into seven major aspects, namely anticancer, anti-inflammatory, anti-oxidative, anti-fibrosis etc. (Fig. 1).

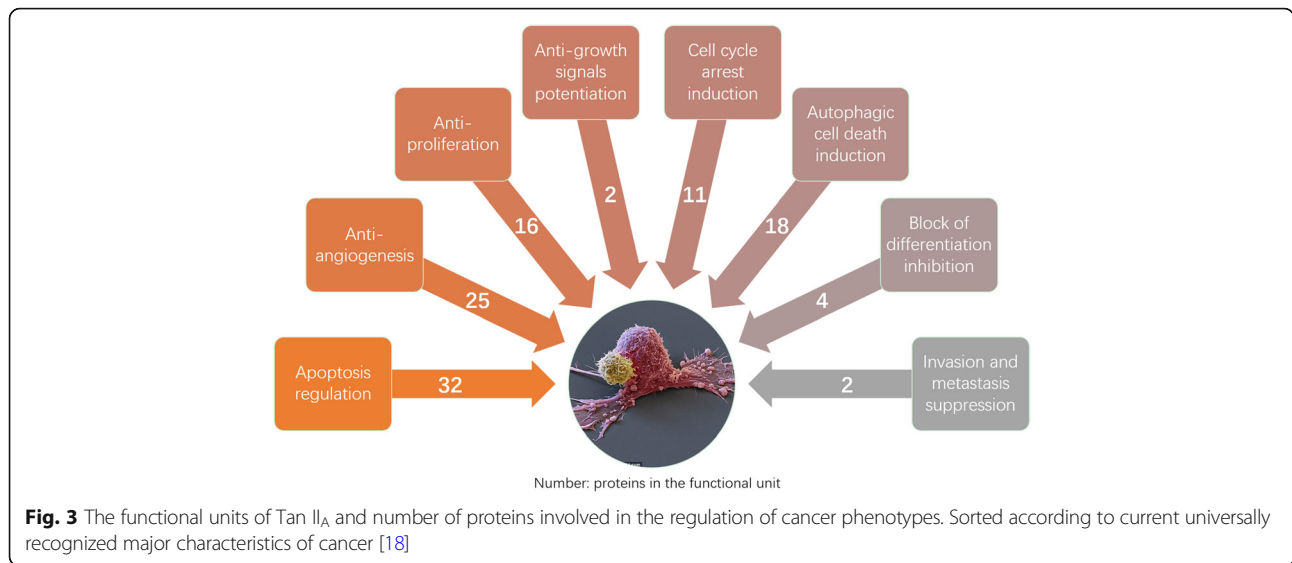### The identification of anticancer network of Tan II$_A$ through pharmacological cluster

The pharmacological cluster on cancers was illustrated (Fig. 2). The node degree distribution followed power law $P(k) = 16.574 \times ^{-0.848}$ (correlation:0.914, R-square:0.800), which indicated that the anticancer network was a scale-free network. A total number of 8 Tan II$_A$ anticancer functional units classified upon the characteristics of cancer they targeted were illustrated [20] (Fig. 3). The present study focused on clusters with a size above 10. The GENEONTOLOGY (GO) enrichment analysis confirmed the clustering results, the biological processes discovered by GO analysis were in consistent with the units (Table 1). All results had *P* value and FDR (False Discovery Rate) lower than 0.05, which indicated they were statistically significant. In topological analysis, the degree of a node was the number of connections it has to other nodes, a node with higher betweenness centrality would have more control over the network [16, 17]. In the present pharmacological cluster, the top 10 nodes identified by Cyto-Hubba were Bcl-2, BCL2 associated X (Bax), B-cell lymphoma-extra large (Bcl-XL), DNA damage inducible transcript 3 (CHOP), induced myeloid leukemia cell differentiation protein (MCL1), translationally controlled tumour protein (TCTP), heat shock protein family A member 5 (BIP), protein kinase B (PKB) also known as

Fig. 2 Tan II$_A$ targets network on cancer created by pharmacological clustering. Analyzed by NetworkAnalyzer. The colour of the node change from blue to red with increased betweenness centrality. The circle size was proportional to the degree of the node. The edge thickness showed degree of connection between two connecting nodes

Akt, phosphoinositide 3-kinase (PI3K) and Fos proto-oncogene, AP-1 transcription factor subunit (Fos).

Apoptosis regulatorypathway analysis of Tan II$_A$

Resistance to apoptosis, an aspect of uncontrolled proliferation which is a distinguishing characteristic of cancer. A functional unit on pathways which regulate apoptosis process was constructed based on literatures referred by ALS (Fig. 4, Additional file 1). The network demonstrated that several signalling pathways were inhibited by Tan II$_A$ simultaneously. On the contrary, Tan II$_A$ up-regulated p38 mitogen-activated protein kinase (MAPK) signalling. Among these proteins, AKT protein in the PI3K/AKT pathway and c-jun N-terminal kinase (JNK) had the highest degree of 7. Moving along the pathway network, Tan II$_A$ elevated the protein expression of transcription factors: activating transcription factor 4 (ATF4), nuclear factor

Fig. 1 Pharmacological clusters and number of proteins regulated by Tan IIA. Sorted by pharmacological clustering method based on function of proteins

Fig. 3 The functional units of Tan II$_A$ and number of proteins involved in the regulation of cancer phenotypes. Sorted according to current universally recognized major characteristics of cancer [18]

(erythroid-derived 2)-like 2 (Nrf2) and CHOP; whereas, it decreased the expression of nuclear factor kappa B (NF-κB) and AP-1. CHOP and NF-κB had the highest degree of 4. The results also showed, in short, that Tan II$_A$ promoted apoptosis by inhibiting the expression of anti-apoptotic regulators and inducing pro-apoptotic regulators. The degrees (7) of anti-apoptotic regulators, Bcl-2 and Bcl-xL, were the highest. The network also revealed that Tan II$_A$ up-regulated proteins which induce apoptosis such as tumour suppressor p53 [19], Bax [20–23] and poly(ADP-ribose) polymerase 1 (PARP1) [22] via both caspase-dependent pathway and caspase-independent pathway. Meanwhile, Tan II$_A$ downregulated inhibitor-of-apoptosis protein (IAP) [24], mediator of the anti-apoptotic signals TNF receptor associated factor (TRAF), cyclin D1 (CCND1) [25] and survivin [26] expression by suppressing PI3K/Akt [27, 28], mitogen-activated protein kinase kinase (MEK)/ extracellular signal–regulated kinase (ERK) [29–31], Janus Kinase (JAK) / Signal Transducer and Activator of Transcription (STAT) [25, 32] and mTOR signalling pathways. Several membrane proteins erb-b2 receptor tyrosine kinase 2 (ERBB2) also known as HER2 [33], epidermal growth factor receptor (EGFR) [28], platelet derived growth factor receptor (PDGFR), TNF receptor superfamily member 10b (DR5) [30] and Fas cell surface death receptor (Fas) [34] were also regulated by Tan II$_A$.

Moreover, Tan II$_A$ increased production of reactive oxygen species which increases oxygen stress in cell and lead to apoptotic cell death.
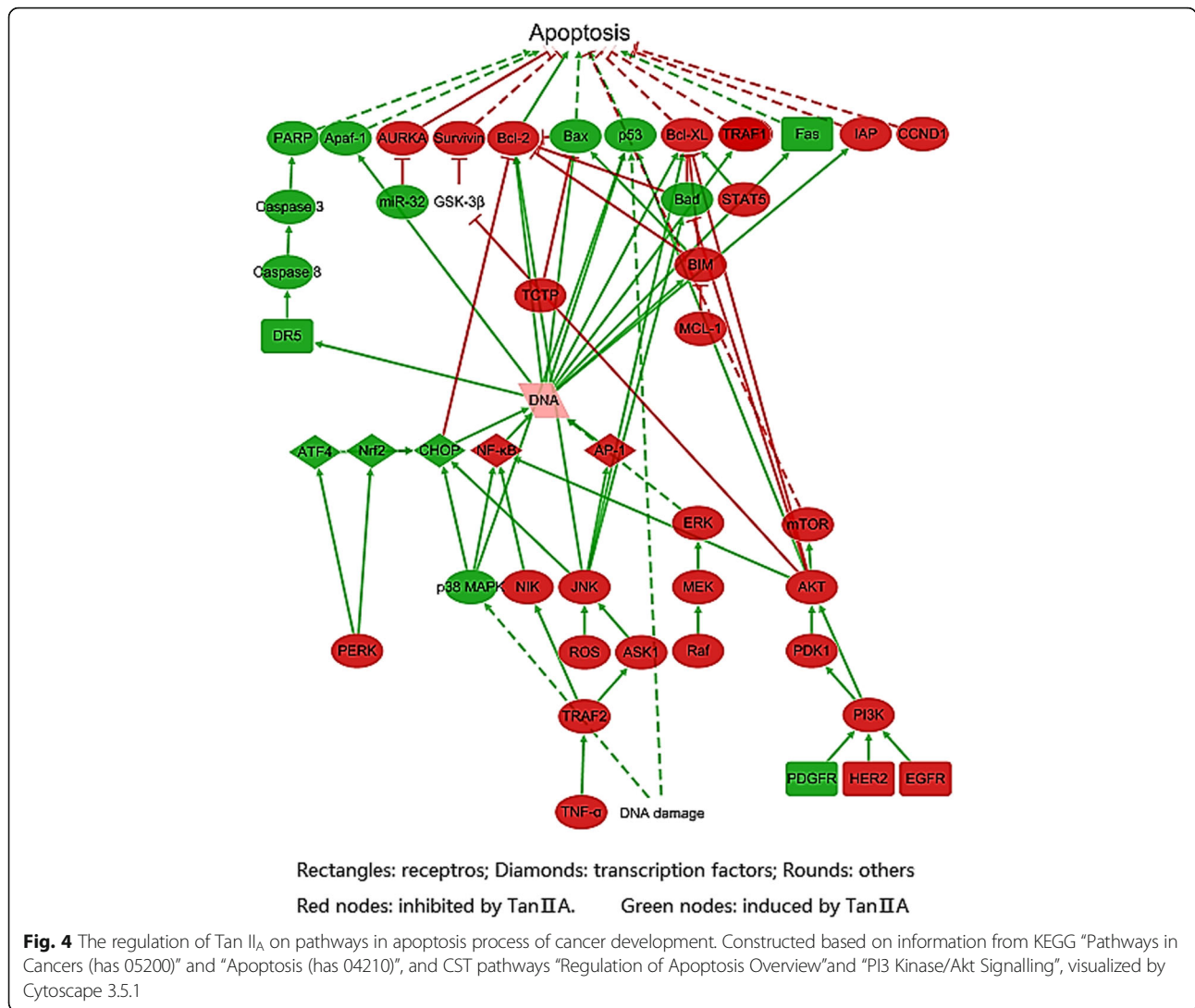
Anti-angiogenesis pathway analysis of tan II$_A$

Sustained angiogenesis is another pathological factor that contributes to uncontrolled proliferation of cancer cells. The pathway network illustrated a part of the systematic regulatory effect of Tan II$_A$ on cancer (Fig. 5). It showed that Tan II$_A$ produced coordinated regulation on sets of functionally related proteins and resulted in an overall anti-angiogenesis effect. Tan II$_A$ inhibited four signalling pathways: PI3K/AKT, JAK/STAT, mTOR and MEK/ERK which in turn down-regulated the expression of three transcription factors: AP-1, HIF-1αand NF-κB. As a result, vascular mediator nitric oxide (NO), matrix metalloproteinase 2 (MMP2), vascular endothelial growth factor (VEGF), cyclooxygenase − 2 and pro-inflammatory cytokine interleukin-8 (IL-8) were inhibited. Among which, VEGF had the highest degree of 6. Meanwhile, mammary serine protease inhibitor (maspin) was up-regulated.

Pathway analysis of anti-proliferation effect on Tan II$_A$

As mentioned above, uncontrolled proliferation is a major characteristic of cancer cell, the inhibitory activity of Tan

Table 1 Validation of networks

NETWORK	FIGURE	GO BIOLOGICAL PROCESS	P VALUE	FDR (FALSE DISCOVERY RATE)
APOPTOSIS REGULATION	4	apoptotic process	2.02E-21	2.43E-18
ANTI-ANGIOGENESIS	5	angiogenesis	1.23E-05	2.74E-02
ANTI-PROLIFERATION	6	cell proliferation	4.56E-04	1.63E-02
CELL CYCLE ARREST	7	regulation of cell cycle	9.87E-06	2.90E-03
AUTOPHAGIC CELL DEATH	8	regulation of autophagy	4.44E-08	6.93E-04

Fig. 4 The regulation of Tan II$_A$ on pathways in apoptosis process of cancer development. Constructed based on information from KEGG "Pathways in Cancers (has 05200)" and "Apoptosis (has 04210)", and CST pathways "Regulation of Apoptosis Overview"and "PI3 Kinase/Akt Signalling", visualized by Cytoscape 3.5.1

II$_A$ on cancer cell proliferation was important to its overall anticancer activity. The pathways of the anti-proliferation mechanisms of Tan II$_A$ was built on evidences provided by ALS (Additional file 1) and information from KEGG and CST pathways (Fig. 6). The diagram showed Tan II$_A$ inhibited two receptors: epithelial growth factor receptor (EGFR) and receptor tyrosine-protein kinase erbB-2 (HER2), it supressed two signalling pathways and down-regulated the expression of proteins that potentiate proliferation such as CCND1, MYC proto-oncogene (c-Myc) [35]. Tan II$_A$ induced interferon alpha-inducible protein 27 (p27) which negatively regulates proliferation.
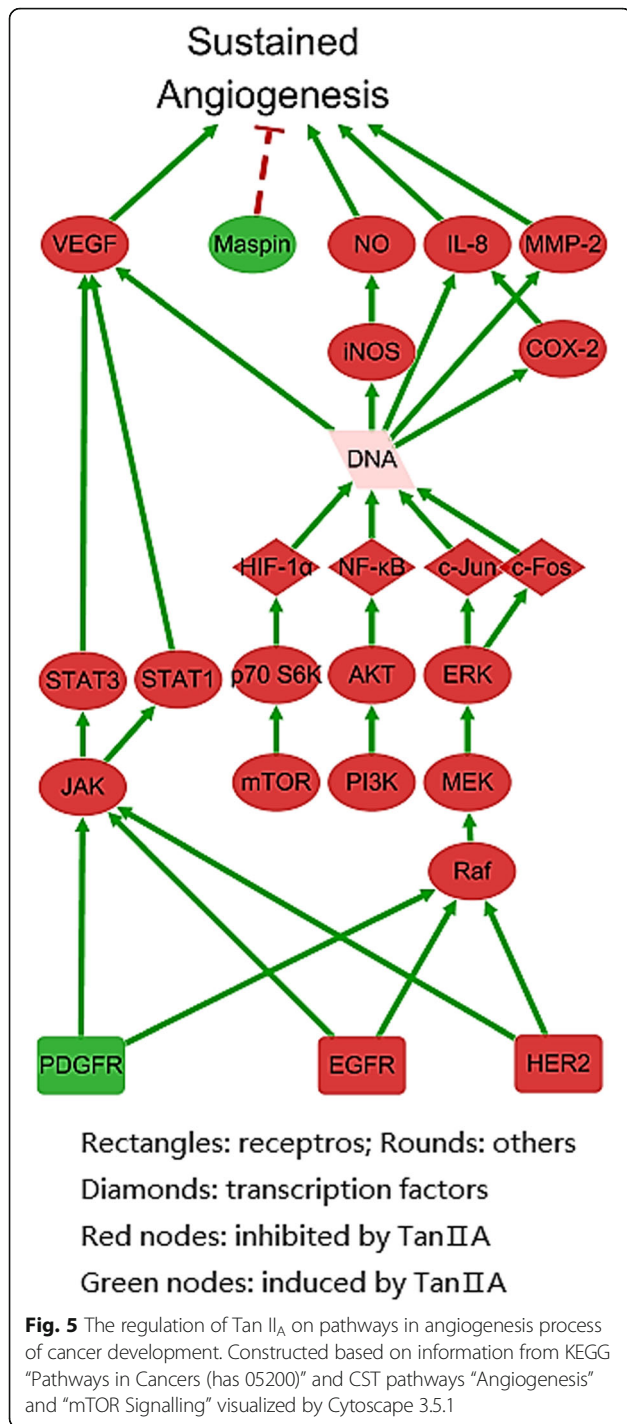
Pathway analysis of carcinoma cell cycle arrest effect of Tan II$_A$

Other than uncontrolled proliferation, another significant features of cancer is dysregulation of cell cycle [36]. As one mechanism evolved in response to DNA damage is halting cell-cycle progression, any alteration would increase the risk

of cancer developing [37]. The pathway network (Fig. 7) was shaped by supporting evidences (Additional file 1) submitted by ALS and information from KEGG and CST pathways. The pathway network revealed the overall inducible effects of Tan II$_A$ on cell cycle arrest. Tan II$_A$ activated phosphorylation of p27; it induced the expression of cyclin-dependent kinase inhibitor 2A (p16) and p53, tumour suppressors, and the expression of cyclin-dependent kinase inhibitor 1 (p21). On the other hand, Tan II$_A$ inhibited the expression of CCND1 and the phosphorylation of cyclin dependent kinase 1 (CDC2).

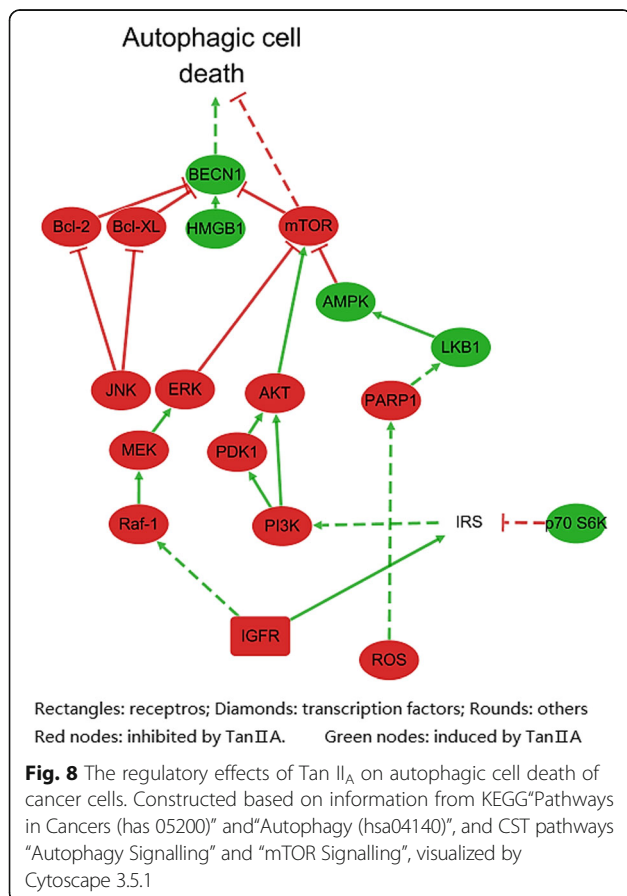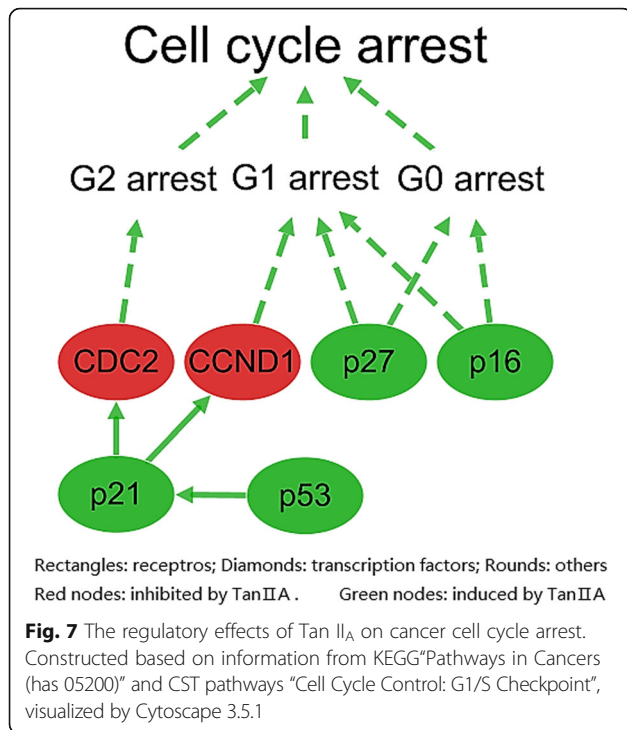## Pathway analysis of autophagic cell death induced by Tan II$_A$

In addition to above anticancer mechanisms, induction of autophagic cell death by Tan II$_A$ also contributed to its anticancer activity. Autophagic cell death is a type of regulated, programmed cell death which is essential to ensure normal cell function. Evidences

Fig. 5 The regulation of Tan II$_A$ on pathways in angiogenesis process of cancer development. Constructed based on information from KEGG "Pathways in Cancers (has 05200)" and CST pathways "Angiogenesis" and "mTOR Signalling" visualized by Cytoscape 3.5.1

Fig. 6 The regulation of Tan II$_A$ on pathways in cancer cell proliferation. Constructed based on information from KEGG "Pathways in Cancers (has 05200)" and CST pathways "PI3 Kinase/Akt Signalling" and "MAPK/ERK in Growth and Differentiation", visualized by Cytoscape 3.5.1

suggested autophagic cell death mediate cancer elimination and that decreased autophagic activity is related to tumorigenesis [38]. Research data on autophagic cell death was referred in ALS supporting evidences, which offered profile for the pathway (Fig. 8). Tan II$_A$ antagonized insulin-like growth factor 1 receptor (IGFR), and decreased production of ROS. Tan II$_A$ induced p70 S6K which indirectly inhibited PI3K/AKT pathway. Meanwhile it suppressed PARP1
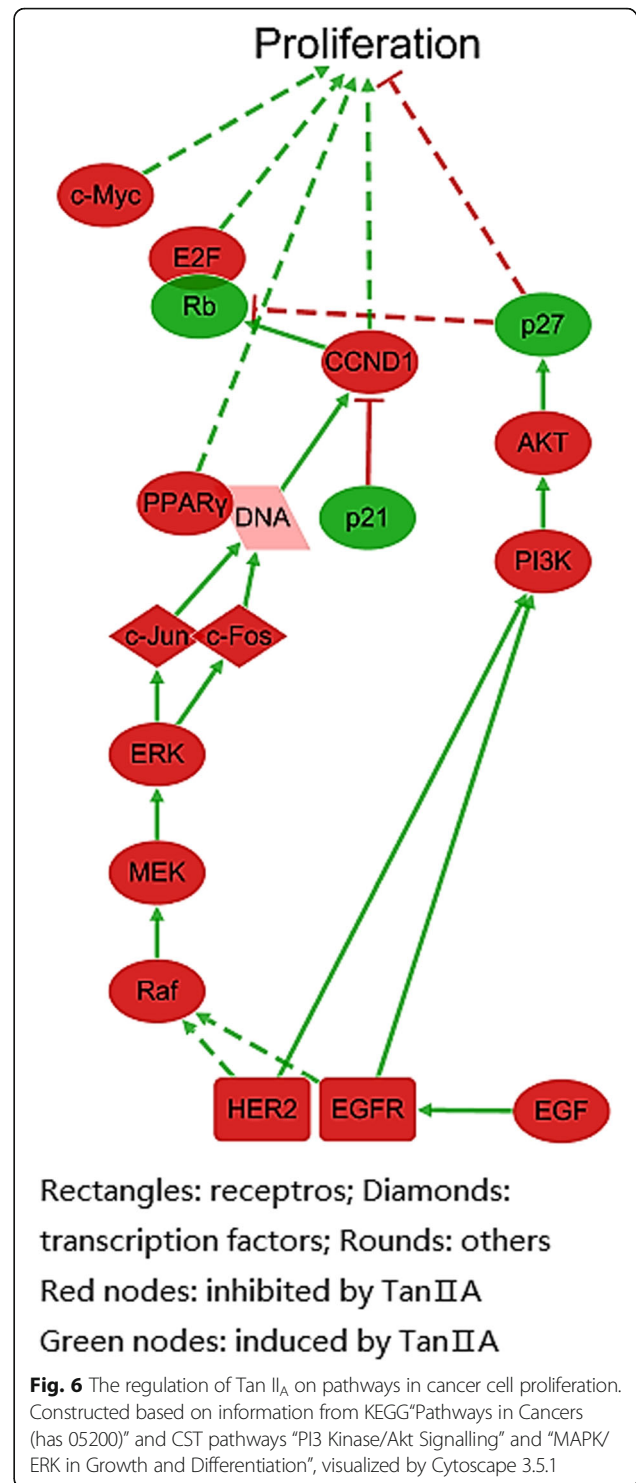
and MEK/ERK signalling pathways simutanously. The regulation on these three pathways resulted in the inhibition of mTOR pathway. Thus induced autophagic cell death, as elevated expression of beclin-1 (BECN1) was observed. Moreover, inhibition of JNK pathway and Bcl-2 and Bcl-xL also contributed to the increase of BECN1.
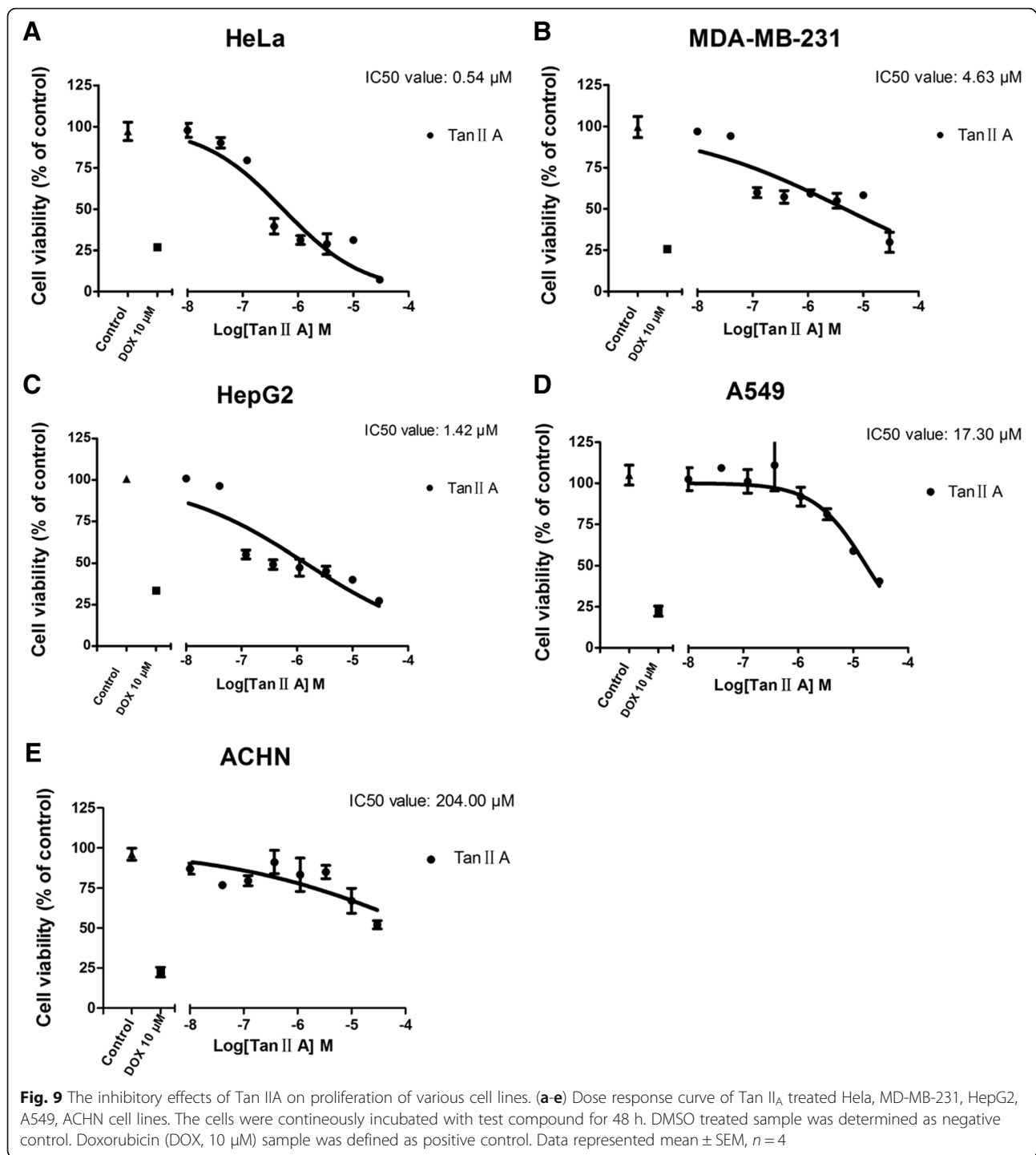
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Fig. 7 The regulatory effects of Tan II$_A$ on cancer cell cycle arrest. Constructed based on information from KEGG "Pathways in Cancers (has 05200)" and CST pathways "Cell Cycle Control: G1/S Checkpoint", visualized by Cytoscape 3.5.1

Fig. 8 The regulatory effects of Tan II$_A$ on autophagic cell death of cancer cells. Constructed based on information from KEGG "Pathways in Cancers (has 05200)" and "Autophagy (hsa04140)", and CST pathways "Autophagy Signalling" and "mTOR Signalling", visualized by Cytoscape 3.5.1

Inhibitory effects of Tan II$_A$ on various carcinoma cell lines

To test the sensitivity and specificity of Tan II$_A$ on various carcinoma cell lines, further assay on cell proliferation was conducted. Tan II$_A$ exhibited dose-dependent inhibitory effects on HeLa, MDA-MB-231, HepG2, A549, and ACHN cells lines. The IC$_{50}$ values were 0.54 μM, 4.63 μM, 1.42 μM, 17.30 μM, and 204.00 μM respectively (Fig. 9a-e). These figures demonstrated that Tan II$_A$ exerted strong selectivity on HeLa cells, moderate effects on MDA-MB-231, HepG2, and A549 cells. While it merely exhibited mild influence on ACHN cells.

Discussion

Cancer is one of the leading causes of morbidity and mortality worldwide and was responsible for around 8.8 million deaths in 2015. Unfortunately, the number of new cases is expected to rise by about 70% over the next two decades [39]. Moreover, the low response rate and tolerance are major concerns of current anti-cancer therapies [40–42]. Therefore, the development of complementary therapeutic interventions is desperately demanded. Danshen has been applied to treat cancerous disease in the history of TCM. One of its major active components, Tan II$_A$, has showed promising effect in in vivo experiments and clinical studies [20, 36, 43]. The compound may influence cancer phenotypes through targeting multiple proteins. Hence the systematic approach developed in the present study fit to delineate the regulatory effects of Tan II$_A$ on cancer-related pathways.

Recent advancements in computational technologies offered powerful tools that can be used to unravel the anticancer effect of Tan II$_A$ in systematic format. Data mining and integration, large scale text-mining though ALS provided a comprehensive evidence-based set of protein targets of Tan II$_A$. The visualization of interactions between the proteins via Cytoscape offered perceivable framework. Topological analysis on protein targets network of Tan II$_A$ identified proteins that had significant influence on the network. However, Tan II$_A$ possesses various pharmacological activities, cardio-protection and anticancer, for example, almost has nothing to do with each other. Therefore, topological analysis on a pack of proteins involved in various activities may somewhat fail to provide specific information. And the structure of topological clusters gathered by ClusterONE is generally susceptible to topological changes, thus the variations may not always represent pharmacological changes. Pharmacological clustering method used in the present study, however, could dramatically improve the robustness of clusters, the resulted anticancer network was a scale-free network with its node degree distribution followed power law, which guaranteed its good robustness and excellent error tolerance. Because power-law distribution implies that the

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Fig. 9 The inhibitory effects of Tan IIA on proliferation of various cell lines. (**a**-**e**) Dose response curve of Tan II$_A$ treated Hela, MD-MB-231, HepG2, A549, ACHN cell lines. The cells were contineously incubated with test compound for 48 h. DMSO treated sample was determined as negative control. Doxorubicin (DOX, 10 μM) sample was defined as positive control. Data represented mean ± SEM, $n = 4$

majority of nodes have only a few links, nodes with small connectivity will be selected with much higher probability. The removal of these 'small' nodes does not alter the path structure of the remaining nodes, and thus has no impact on the overall communication effectiveness of the anticancer network [44]. Moreover, this approach helped to draw focus on the analysis of a specific bioactivity, take the anticancer effect for example. Further pathway analysis

provided deep insight into five refined functional units of anticancer activity and shed new light on the molecular mechanisms of Tan II$_A$. These mined targets data and pathway networks offered a systematic approach to unravel the anticancer mechanisms of Tan II$_A$ and its effect on tumour microenvironment.

Tumour microenvironment describes the non-cancerous cells present in the tumour [45]. Increasing evidence

suggests that the tumour microenvironment are quite complex, which includes fibroblasts and myofibroblasts, neuroendocrine cells, adipocytes, immunological and inflammatory cells, the blood and lymphatic vascular networks, and extracellular matrix [18]. Pharmacological clustering method identified the protective effects of Tan II$_A$ against fibrosis diseases, inflammatory responses, and oxidative stress. These effects jointly play a positive role in improving tumour microenvironment.

The functional units of Tan II$_A$ overlapped on signalling proteins, several proteins in signalling pathways, for instance MAPK signalling pathway and JAK/ STAT signalling pathway, were involved in multiple functional units and counted more than once. The large size (32 nodes) of the pathway network (Fig. 3) indicated that Tan II$_A$ regulated multiple apoptosis pathways simultaneously and exerted its anticancer activity mainly through inhibiting anti-apoptosis of carcinoma cells. The MCC analysis suggested Bcl-2, Bcl-xL, and CHOP played key role in the network. These proteins are considered critical therapeutic targets of Tan II$_A$ for anticancer therapy. The result revealed the importance of VEGF in sustained angiogenesis pathology. Tan II$_A$ down-regulated this growth factor through four pathways and it showed highest degree in the network. It is notable that the pathway network suggested Tan II$_A$ up-regulated PDGFR to promote vascular normalization therefore inhibited metastasis. The functional unit on carcinoma cell proliferation revealed that Tan II$_A$ acted contrary to proteins in the same pathway, it inhibited PI3K/AKT, whereas induced the down-stream effector p27. The contradictory results proposed the possibility of a direct action of Tan II$_A$ on p27. The inducible effect of Tan II$_A$ on autophagic cell death was mainly executed by inhibition of mTOR pathway. The significance of mTOR was verified by its high degree of connection in the present pathway analysis.

In addition to the five functional units aforementioned, Tan II$_A$ also regulated several other units that have not been illustrated for the sake of their limited size. It inhibited SMAD family member (Smad) 2 and 3 to attenuate insensitivity of cancer cell to anti-growth signals. The de-differentiation of cancer cell was inhibited through inhibition of IL-6, CCAAT/enhancer binding protein alpha (CEBPα) [46], Spi-1 proto-oncogene (PU.1), c-Myc and E2F transcription factor 1 (E2F1). Moreover, Tan II$_A$ inhibited NF-κB signalling and expression of MMP-2 and MMP-9, and increased levels of tissue inhibitor of matrix metalloproteinases type 1 and 2 (TIMP-1 and TIMP-2) [47], therefore suppressed invasion and metastasis of cancer cells.

The results of cell experiments announced the selectivity of Tan II$_A$ on different cancer cell lines. It showed that HeLa and MDA-MB-231 were more sensitive to Tan II$_A$ treatment than A549 and ACHN. Which

confirmed the traditional uses of Danshen in gynaecology recorded in historical bibliographies such as The Great Pharmacopoeia and the Complete Book of Good Prescriptions for Women. Moreover, HepG2 was found to be sensitive to Tan II$_A$ treatment as well. This finding also shed new light on the morden therapeutic use of Danshen on hepatic disorders.

Conclusions

Cancer is one of the leading causes of death globally. Which has been recognized as a Systems Biology disease [48]. Therefore, a network-based approach has been applied in the present study to delineate the anticancer mechanism of Tan II$_A$, the major active component of a Chinese medicinal herb: Danshen. We constructed pharmacological clustering method to focus network analysis on the pharmacological actions of Tan II$_A$ instead of the topological features of network. Thereby the network analysis revealed that Tan II$_A$ produced its anticancer effect mainly through induction of carcinoma cell apoptosis, cell cycle arrest and autophagic cell death; anti-proliferation and anti-angiogenesis. The study confirmed that Tan II$_A$ systematically regulated molecular and protein events and cancer micro-environment to produce its anticancer effect. The cell experiments indicated the selectivity of Tan II$_A$ towared HeLa, HepG2 and MDA-MB-231 cell lines.

The present study offered an innovative approach for a comprehensive pathway study on the anticancer effect of Tan II$_A$. The methodology constructed can be applied to a broad-spectrum of medicinal herbs and active compounds. It provided a novel researching method for actions of traditional Chinese medicinal herbs through protein networks in future. It focused not only on single target but also on several pathways that participated in the disease progression, and regulations of the herbal component on these pathways. As the concept of this approach is in consistent with the guiding theory of TCM that "the whole defines parts", it proved to be a suitable method for TCM studies. The method offered guidance for further experiments which could hopefully minimize the suffering and sacrifices of experimental animals. As the current study mainly reveal the molecular mechanisms of Tan II$_A$ through computational-based network pharmacological method, further experiments are still needed for confirming the regulatory effects of the mined protein targets and pathways.

Methods

Data mining

Cytoscape app "Agilent Literature Search" (ALS) software was used for data mining, it is a meta-search tool for automatically querying multiple text-based search engines (both public and proprietary) when a query was

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entered [49]. Query "Tanshinone II$_A$" was entered, context was set to "blank", maximum number of engine matches was set to 1000, interaction lexicon was set to "limited" and all other parameters were set to default. Obtained hits were checked against supporting evidences generated automatically during the search, false positives were removed.

Pharmacological clustering

A clustering method, pharmacological clustering, based on physiological and/or pathological function of proteins identified by ALS was conducted. The clustering was based on information of genes and their coded protein which were gathered from NCBI databases and literatures. The anticancer module was obtained. The node degree distribution of anticancer network was analysed. Then proteins in the module were put into functional units according to ALS supporting evidences: cancer – apoptosis, cancer – proliferation, cancer – cell cycle, cancer – angiogenesis, cancer – differentiation and others. For instance, NCBI gene database indicated that BCL2 associated X (Bax) is involved in cancer cell apoptosis regulation, hence it was put in the pharmacological cluster cancer – apoptosis; whereas AKT serine/threonine kinase 1 (AKT), a component of PI3K/AKT signalling pathway, was included in several pharmacological clusters, such as cardio-protection and anti-inflammation, as evidences suggested that it is involved in various cellular processes. The clustering results were validated by PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships) [50] facilitated GO enrichment analysis. The protein specific UniProt ID of each target was obtained and processed for GO enrichment analysis for each cluster.

CytoHubba, a Cytoscape plug-in, was utilized to explore the important nodes/hubs in the ALS-constructed Tan II$_A$ targets network on cancer. Matthews Correlation Coefficient (MCC) assessment was chosen as the ranking method, and ten top-ranking nodes were identified [51]. Other parameters were set to default values.

Recognition and network construction of anticancer pathways regulated by Tan II$_A$

The anticancer pathways regulated by Tan II$_A$ were found by combining information obtained from KEGG and Cell Signalling Technology (CST) Pathways, the relations (induction and inhibition) between targets of Tan IIA were extracted. KEGG pathways "Pathways in Cancers (has 05200)", "Apoptosis (has 04210)" and "Autophagy (hsa04140)"; CST pathways "Regulation of Apoptosis Overview", "Autophagy Signalling", "Angiogenesis", "Cell Cycle Control: G1/S Checkpoint", "PI3 Kinase/Akt Signalling", "MAPK/Erk in Growth and Differentiation" and "mTOR Signalling" were used to delineate anticancer pathways regulated by Tan II$_A$. Then the pathways were plotted using Cytoscape 3.5.1. The degree of a node in a pathway network was calculated by counting the number of connections it had.

Cell proliferation evaluation by CCK-8 assay

Cell culture Human non-small-cell lung cancer cell line A549, human kidney cancer cell line ACHN, human hepatocellular carcinoma HepG2 and human triple-negative breast cancer cell line MDA-MB-231 were derived from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM; HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Shanghai, China) and 1% Penicillin-Streptomycin solution. Human cervical cancer cell line HeLa was cultured in RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin solution. The cell lines were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Tanshinone II$_A$ (M.W. = 328, T4952, ≥97% -HPLC) was purchased from Jiangxi herbfine Co. Ltd. (CAS No. 568–72-9).

Cytotoxicity of Tan II$_A$ was analysed by cell counting kit-8 (CCK-8; Biodee Biotechenology, Beijing, China) in A549, ACHN, MDA-MB-231, HepG2 and HeLa cell lines. The Dojindo's highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] contained in CCK-8 solution produces a water-soluble formazan orange colour dye upon reduction. The assay is based on this reduction of WST-8 by dehydrogenases in living cells. The amount of the formazan dye generated is directly proportional to the number of living cells. Cells for CCK8 assay were seeded in a 96-well-microplate at a density of 3600 cells/well and treated with various concentrations of tanshinone II$_A$ for 48 h. Control groups were treated with 10 μM doxorubicin at the same incubation environment for 48 h. After the treatment, CCK-8 solution (10 μL) was added to each well, the plate was then incubated for 2 h before the absorbance was measured at 450 nm using a microplate reader.

Statistical analyses

The in vivo experiment data were presented as mean ± standard deviation. The experiments were conducted independently for at least three times. Dose-response curves and IC$_{50}$ values were drawn and calculated by Graphpad prism (version 5, GraphPad Software, Inc., Carlifonia).

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Additional file

Abbreviations

Akt: protein kinase B; ALS: Agilent Literature Search; ATF4: activating transcription factor 4; Bax: BCL2 associated X; Bcl-xL: B-cell lymphoma-extra large; BECN1: beclin-1; BIP: heat shock protein family A member 5; CCND1: cyclin D1; CDC2: cyclin dependent kinase 1; CEBPα: CCAAT/enhancer binding protein alpha; CHOP: DNA damage inducible transcript 3; c-Myc: MYC proto-oncogene; CST: Cell Signaling TECHNOLOGY; DR5: TNF receptor superfamily member 10b; E2F1: E2F transcription factor 1; EGFR: epidermal growth factor receptor; ERK: extracellular signal–regulated kinases; Fas: Fas cell surface death receptor; Fos: Fos proto-oncogene, AP-1 transcription factor subunit; HER2: erb-b2 receptor tyrosine kinase 2; IAP: inhibitor-of-apoptosis protein; IGFR: insulin-like growth factor 1 receptor; IL-8: interleukin-8; JAK: Janus Kinase; JNK: c-jun N-terminal kinase; Maspin: mammary serine protease inhibitor; MCL1: induced myeloid leukemia cell differentiation protein; MEK: mitogen-activated protein kinase kinase; MMP2: matrix metalloproteinase 2; mTOR: mechanistic target of rapamycin; NCBI: National Center for Biotechnology Information; NF-κB: nuclear factor kappa B; NO: nitric oxide; NRF2: nuclear factor (erythroid-derived 2)-like 2; p16: cyclin-dependent kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1; p27: interferon alpha-inducible protein 27; p38 MAPK: p38 mitogen-activated protein kinase; p70 S6K: ribosomal protein S6 kinase B1; PARP1: poly(ADP-ribose) polymerase 1; PDGRF: platelet derived growth factor receptor; PI3K: phosphoinositide 3-kinase; PU.1: Spi-1 proto-oncogene; Smad: SMAD family member; STAT: Signal Transducer and Activator of Transcription; Tan II$_A$: Tanshinone II$_A$; TCM: traditional Chinese medicine; TCTP: translationally controlled tumour protein; TIMP-1: matrix metalloproteinases type 1; TIMP-2: matrix metalloproteinases type 2; TRAF: TNF receptor associated factor; VEGF: vascular endothelial growth factor

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the KEGG repository, http://www.kegg.jp/kegg/kegg2.html; Cell Signaling TECHNOLOGY repository, https://www.cellsignal.com/ and Gene Ontology enrichment analysis, http://geneontology.org/page/go-enrichment-analysis.

Authors' contributions

YQ and YZ conceived the work. YC conducted data mining and analysed the results. YC, SW and XL conducted cell experiments. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable. The manuscript does not report on or involve the use of any animal or human data or tissue.

Consent for publication

Not applicable. The manuscript does not contain data from any individual person.

Competing interests

The authors declare that they have no competing interests.

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References

1. Wang D, Wang L. Study on the anti-cancer effects of Huoxue Huayu Chinese herbs. Jilin J Tradit Chin Med. 1993;1:44–5.
2. Lin M. Contribution of Shennong's Classic of Materia Medica on the development of Anti-cancer Chinese medicinal products [D]. Guang Zhou University of Chinese Medicine; 2011. Chinese.
3. Qing-lin W, Li X. Law research on Chinese medicine with prescription anti-cancer proprietary. Chin J Exp Tradit Med Formula. 2013;19(13):356–9.
4. Liao J, Zhao M, Li Y. A review on pharmacological actions of Danshen. Contemp Med. 2017;23(27):182–4.
5. Wu J, Liu S, Zhang X, Zhang B. Danshen injection as adjuvant treatment for unstable angina Pectoris : a systematic review and meta-analysis. Chin J Integr Med. 2017;23(4):306–11. https://doi.org/10.1007/s11655-016-2272-0.
6. Chen X, Guo J, Bao J, Lu J, Wang Y. The anticancer properties of Salvia Miltiorrhiza Bunge (Danshen): a systematic review. Med Res Rev. 2013; 161(4):768–94. https://doi.org/10.1002/med.
7. Bae W, Choi J, Kim K, Syn HU. Inhibition of proliferation of prostate cancer cell line DU-145 in vitro and in vivo using Salvia miltiorrhiza Bunge. Chin J Integr Med. 2017; Epub ahead.
8. Wu C, Cherng J, Yang Y, Lin C. Danshen improves survival of patients with advanced lung cancer and targeting the relationship between macrophages and lung cancer cells. Oncotarget. 2017;8(53):90925–47.
9. Kaptchuk TJ. Chinese Medicine: The Web That Has No Weaver. 2nd ed. New York: Rider; 2000.
10. Zhang Y-Q, Mao X, Guo Q-Y, Lin N, Li S. Network pharmacology-based approaches capture essence of Chinese herbal medicines. Chinese Herb Med. 2016;8(2):107–16. https://doi.org/10.1016/S1674-6384(16)60018-7.
11. Agarwal P, Searls DB. Literature mining in support of drug discovery. Br Bioinform. 2008;9(6):479–92. https://doi.org/10.1093/bib/bbn035.
12. Khatri P, Sirota M, Butte AJ. Ten years of pathway Analysis : current approaches and outstanding challenges. PLoS Comput Biol. 2012;8(2):1–10. https://doi.org/10.1371/journal.pcbi.1002375.
13. UK Cancer incidence for common cancers [homepage on the Internet]. London: Cancer Research UK. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics-for-the-uk#collapseZero. Accessed 15 Dec 2017.
14. American Cancer Society. Cancer Facts and Figures 2017. Atlanta, Ga: American Cancer Society; 2017.
15. Li X, Du J, Bai B, et al. Inhibitory effects and mechanism of tanshinone IIA on proliferation of rat aortic smooth muscle cells. Zhongguo Zhong Yao Za Zhi. 2008;33(17):2146–50.
16. Newman M. Networks: an introduction. Oxford: Oxford University press; 2010.
17. Newman M. A set of measures of centrality based on betweenness. Soc Netw. 2005;27(1):39–54.
18. Wang M, Zhao J, Zhang L, et al. Role of tumor microenvironment in tumorigenesis. J Cancer. 2017;8:761–73. https://doi.org/10.7150/jca.17648.
19. Luo Q, Beaver JM, Liu Y, Zhang Z. Dynamics of p53 : a master decider of cell fate. Genes (Basel). 2017;8(66):1–16. https://doi.org/10.3390/genes8020066.
20. Chien S, Kuo S, Chen Y, Chen D. Tanshinone IIA inhibits human hepatocellular carcinoma J5 cell growth by increasing Bax and caspase 3 and decreasing CD31 expression in vivo. Mol Med Rep. 2012;5(1):282–6. https://doi.org/10.3892/mmr.2011.631.
21. Su CC. Tanshinone IIA inhibits human gastric carcinoma AGS cell growth by decreasing BiP, TCTP, mcl - 1 and Bcl - xL and increasing Bax and CHOP protein expression. Int J Mol Med. 2014:1661–8. https://doi.org/10.3892/ijmm.2014.1949.
22. Liu F, Yu G, Wang G, et al. An NQO1-initiated and p53-independent apoptotic pathway determines the anti-tumor effect of Tanshinone IIA against non-small cell lung Cancer. PLoS One. 2012;7(7):e42138. https://doi.org/10.1371/journal.pone.0042138.
23. Wang J, Feng J, Han J, Zhang B, Mao W. The molecular mechanisms of Tanshinone IIA on the apoptosis and arrest of human esophageal carcinoma cells. Biomed Res Int. 2014:1–9. https://doi.org/10.1155/2014/582730.
24. Yun S, Jeong S, Kim J, et al. Activation of c-Jun N-terminal kinase mediates Tanshinone IIA-induced apoptosis in KBM-5 chronic myeloid leukemia cells. Biol Pharm Bull. 2013;36(2):208–14.

25. Tang C, Xue H, Huang H, Wang X. Tanshinone IIA inhibits constitutive STAT3 activation , suppresses proliferation , and induces apoptosis in rat C6 glioma cells. Neurosci Lett. 2010;470:126–9. https://doi.org/10.1016/j.neulet.2009.12.069.

26. Liu P, Xu S, Zhang M, et al. Anticancer activity in human multiple myeloma U266 cells: synergy between cryptotanshinone and arsenic trioxide. Metallomics. 2013;5(7):871–8. https://doi.org/10.1039/c3mt20272k.

27. Won S, Lee H, Jeong S, et al. Tanshinone IIA induces mitochondria dependent apoptosis in prostate Cancer cells in association with an inhibition of phosphoinositide 3- kinase / AKT pathway. Biol Pharm Bull. 2010;33(11):1828–34.

28. Su C, Chiu T. Tanshinone IIA decreases the protein expression of EGFR , and IGFR blocking the PI3K / Akt / mTOR pathway in gastric carcinoma AGS cells both in vitro and in vivo. Oncol Rep. 2016;36(2):1173–9. https://doi.org/10.3892/or.2016.4857.

29. Chen W, Liu L, Luo Y, et al. Cryptotanshinone activates p38/JNK and inhibits Erk1/2 leading to caspase-independent cell death in tumor cells. Cancer Prev Res. 2012;5(5):778–87. https://doi.org/10.1158/1940-6207.CAPR-11-0551.

30. Chang C, Kuan C, Lin J, Lai J, Ho T. Tanshinone IIA facilitates TRAIL sensitization by up-regulating DR5 through the ROS-JNK-CHOP signaling Axis in human ovarian carcinoma cell lines. Chem Res Toxicol. 2015;28(8):1574–83. https://doi.org/10.1021/acs.chemrestox.5b00150.

31. Ip Y, Davis R. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol. 1998;10(2):205–19.

32. Jung JH, Kwon T, Jeong S, et al. Apoptosis induced by Tanshinone IIA and Cryptotanshinone is mediated by distinct JAK / STAT3 / 5 and SHP1 / 2 signaling in chronic myeloid leukemia K562 cells. Evid Based Complement Altern Med. 2013;2013:1–10. Article ID 805639, http://dx.doi.org/10.1155/2013/805639.

33. Su C, Lin Y. Tanshinone IIA down-regulates the protein expression of ErbB-2 and up-regulates TNF- α in colon cancer cells in vitro and in vivo. Int J Mol Med. 2008;22(6):847–51. https://doi.org/10.3892/ijmm.

34. Paper O. Growth inhibition and apoptosis induction by Tanshinone IIA in human Colon adenocarcinoma cells. Planta Med. 2008;74:1357–62. https://doi.org/10.1055/s-2008-1081299.

35. Niu Z, Liu H, Zhou M. Knockdown of c-Myc inhibits cell proliferation by negatively regulating the Cdk/Rb/E2F pathway in nasopharyngeal carcinoma cells. Acta Biochim Biophys Sin. 2015;47(3):183–91.

36. Chiu SC, Huang SY, Chen SP, Su CC, Chiu TL, Pang CY. Tanshinone IIA inhibits human prostate cancer cells growth by induction of endoplasmic reticulum stress in vitro and in vivo. Prostate Cancer Prostatic Dis. 2013;16(4):315–22. https://doi.org/10.1038/pcan.2013.38.

37. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004;432:316–23.

38. Shimizu S, Yoshida T, Tsujioka M, Arakawa S. Autophagic cell death and Cancer. Int J Mol Sci. 2014;15(2):3145–53. https://doi.org/10.3390/ijms15023145.

39. WHO. WHO Cancer Fact sheet. http://www.who.int/mediacentre/factsheets/fs297/en/. Accessed 04 Sept 2017.

40. Kumar A, Soares HP, Balducci L, Djulbegovic B. Treatment tolerance and efficacy in geriatric Oncology : a systematic review of phase III randomized trials conducted by five National Cancer Institute – sponsored cooperative groups. J Clin Oncol. 2018;25(10):7–9.

41. Harris AL, Hochhauser D. Mechanisms of multidrug resistance in Cancer treatment. Acta Oncol (Madr). 1992;31(January):205–13.

42. Kelderman S, Schumacher TNM, Haanen JBAG. Acquired and intrinsic resistance in cancer immunotherapy. Mol Oncol. 2014;8:1132–9.

43. Lin C, Wang L, Wang H, Yang L, Guo H, Wang X. Tanshinone IIA inhibits breast Cancer stem cells growth in vitro and in vivo through attenuation of IL-6/STAT3/NF-kB signaling pathways. J Cell Biochem. 2013;114(9):2061–70. https://doi.org/10.1002/jcb.24553.

44. Albert R, Jeong H, Barabasi A. Error and attack tolerance of complex networks. Nature. 2000;406:378–82.

45. Nature.com, Cancer microenvironment. https://www.nature.com/subjects/cancer-microenvironment. Accessed 18 Dec 2017.

46. Rhiu S, Chae MK, Lee EJ, Lee JB, Yoon JS. Effect of Tanshinone IIA in an in vitro model of graves ' Orbitopathy. IOVS. 2017;55(9):5900–10. https://doi.org/10.1167/iovs.14-14008.

47. Shan Y, Shen X, Xie Y, et al. Inhibitory effects of tanshinone II-A on invasion and metastasis of human colon carcinoma cells. Acta Pharmacol Sin. 2009;30(11):1537–42. https://doi.org/10.1038/aps.2009.139.

48. Hornberg JJ, Bruggeman FJ, Westerhoff HV, Lankelma J. Cancer : a systems biology disease. Biosystems. 2006;83:81–90. https://doi.org/10.1016/j.biosystems.2005.05.014.

49. Creech M, Kuchinsky A, Vailaya A. Agilent Literature Search Software. 2014.

50. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. Nat Protoc. 2013;8:1551–66.

51. Shao-jun C. Drug-target networks for Tanshinone IIA identified by data mining. Chin J Nat Med. 2015;13(10):751–9. https://doi.org/10.3724/SP.J.1009.2015.00751.