Tanshinone IIA Ameliorates H_2O_2-induced Antioxidant Effects in Huh7 Cells

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Abstract. Objective This study aims to elucidate the antioxidative mechanisms of tanshinone IIA (Tan IIA) in ameliorating hydrogen peroxide (H_2O_2)-induced oxidative stress in the Huh7 cellular model. **Methods** The association between Tan IIA and HCC was analyzed using network pharmacology techniques. Changes in Tan IIA activity on H_2O_2-induced Huh7 cells and related antioxidant indices were determined using CCK-8. The cells underwent analysis using Western blot and immunofluorescence assessments to delineate the concentrations of PI3K/Akt and Nrf2/HO-1 proteins and their corresponding interactions. **Results** Network pharmacological analysis demonstrated the multi-targeting properties of Tan IIA for the treatment of HCC. The oxidative stress state was significantly alleviated with the intervention of Tan IIA, and the cellular levels of the superoxide dismutase (SOD) was elevated, malondialdehyde (MDA) level was decreased, and glutathione peroxidase (GSH-Px) was elevated. Meanwhile, Western blot and immunofluorescence experiments revealed that Tan IIA promoted the activity of Nrf2 and HO-1 proteins, ameliorated oxidative stress phenomenon, with the inhibitory effect on phosphorylation of PI3K and Akt. **Conclusions** Our investigation indicates that Tan IIA modulates HCC through suppression of PI3K/Akt and stimulation of Nrf2/HO-1 signaling cascade.

Keywords: Tanshinone IIA; Antioxidant; PI3K/Akt Signaling Pathway; Nrf2/HO-1 Signaling Pathway; Hepatocellular Carcinoma

1. Introduction

Hepatocellular carcinoma (HCC) composes approximately 75% to 85% of all primary liver carcinomas [1]. More than 2/3 of liver cancer sufferers are commonly in intermediate to advanced phase upon initial diagnosis, and radical interventions such as surgical resection, liver transplantation [2]. Traditional Chinese medicine (TCM), boasting the merits of minimizing toxicity, possessing a superior safety margin, has greatly contributed to the management of HCC [3]. Tanshinone IIA (Tan IIA), sourced from *Salvia miltiorrhiza*, exhibits hepatoprotective, antioxidant, anti-inflammatory, and immune-modulatory effects. It has been reported to suppress HCC by interfering with PI3K/Akt signaling pathway [4], is capable of mitigating damage by stimulating Nrf2 pathway [5]. Oxidative stress damage denotes the manifestation whereby intracellular levels of reactive oxygen species (ROS) exceed cell's antioxidant defense mechanism. This is a prevalent form of stress injury, capable of inducing cellular degeneration, and serves as a significant mechanism and association in the advancement of HCC [6]. Nrf2 represents a pivotal regulatory factor in upholding redox homeostasis [7], serving a vital role in HCC. Moreover, ROS originating from an imbalanced intercellular oxidant and antioxidant spectrum can also impact cell proliferation and survival via controlling PI3K/Akt signaling cascade [8]. In the current research endeavor, it was conjectured that Tan IIA might efficaciously mitigate HCC through means of PI3K/Akt as well as Nrf2/HO-1 regulatory pathways.

2. Materials and Methods

2.1 Materials

Tan IIA was procured from Push Biotechnology (Chengdu, China). The Cell Counting kit-8 (CCK-8) were acquired from Beyotime Biotechnology (Shanghai, China). The kits pertinent to the quantification of the malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). GAPDH, PI3K, p-PI3K, AKT, p-AKT from the Cell Signaling Technology (Danvers, USA). Nrf2 and HO-1 from Abcam (Abcam, USA).

2.1.1 Target Prediction of Tan IIA Chemical Constituents

All chemical components of Tan IIA were searched in TCM Systematic Pharmacology Database (TCMSP, http://lsp.nwu.edu.cn/tcmsp). The requisite for an oral bioavailability (OB) ≧ 30%, drug-like index (DL) ≧0.18 for the initial evaluation of prospective active ingredients. Corresponding target proteins were extracted from the TCMSP database.

2.1.2 Construction of a Database of Potential HCC Targets

HCC associated targets were from: A Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org/), a TTD database (https://db.idrblab.org/ttd). a GeneCards database (https://www.genecards.org/) and a Disgenet database (http://www. disgenet.org/ web/ DisGeNET/ menu/home). Subsequently, these HCC associated genes underwent standardization using a UniProt database (https://www.uniprot.org/). Association with crossover genes was confirmed using R software utilizing the Venn Diagram module.

2.1.3 Protein-protein Interaction Data

A STRING database (https://string-db.org/) was leveraged to identify potential protein-protein interactions (PPIs). PPI data were examined using STRING with species restricted to "Homo sapiens" and a reliability threshold greater than 0.4, and the remaining PPIs were used for building and examining networks. Cytoscape software (version 3.7.2) can be used to apply to view and merge parameters of PPI networks related to the principal components of HCC management.

2.1.4 Detection of Enrichment in Gene Ontology (GO)

It classifies these entities under three primary hierarchical facets: molecular function (MF), cellular component (CC) as well as biological process (BP). Each tier incorporates numerous terms to articulate diverse facets of genes and gene products.

2.2 Methods

2.2.1 Cell Cultivation and Cell Viability Assay

Huh7 cells were kept in a moisture-controlled environment at temperature of 37°C and 5% CO_2 concentration. These cells were then kept in the plates and incubated for 24h, subsequent to the application of Tan IIA (0, 0.25, 0.5, 1, 2, 4, 8, 16 μM) for 24h cultivation or H_2O_2 (0, 50, 100, 150, 200, 250, 300 μM) for 2h. CCK-8 solution was supplemented and the examination was executed.

2.2.2 H_2O_2-induced Oxidative Damage Cell Models and Treatment

Huh7 cells at the logarithmic development stage were plated and incubated for 24 hours. Subsequently, the culture medium was aspirated and the cells were classified into six groups as follows: (i) control group; (ii) model group: H_2O_2-infused group. (iii)(iv)(v) the Tan IIA group: incomplete media containing 1, 2 and 4 μM of Tan IIA. (vi) the colchicine group: 1 μM. Subsequently, that medium were exchanged in the Tan IIA and colchicine group and the model group by replacing it with 200 μM H_2O_2 medium and medium was added to both the experimental and control group,

constituting an equivalent volume. Further, the cells were allowed to further incubate for 2h. Subsequently, CCK-8 solution was added and the assay was executed.

2.2.3 Measurement of MDA, SOD, GSH and GSH-Px

Cells underwent centrifugation at 12000 rpm and 4 °C for a duration of 10 minutes, after which the relevant chemical agents were used to measure concentrations of MDA, SOD, and GSH-Px inside cells. The parameters were ascertained according to the guidelines specified in the kit.

2.2.4 Immunofluorescence Staining

The cultured cell lines were attached to coverslips, washed with PBS, stabilized in 4% paraformaldehyde for 10 minutes. Slides underwent permeabilization using 0.5% Triton X-100 for 20 minutes, washed with PBS. The cells underwent blockage using 5% BSA for 1h at room temperature, incubated with Nrf2 antibody or HO-1 antibody overnight. Then incubated using secondary antibodies. The nuclei were stained with Hoechst for 20 minutes.

2.2.5 Western Blot Analysis

The initial protein extraction underwent subsequent 10% SDS-PAGE gel electrophoresis prior to its transfer onto a PVDF membrane. The membrane was immersed in a formulated solution comprising 5% BSA at ambient temperature for 1h. Subsequently, the membranes were subjected to specific primary antibodies inclusive of Nrf2 (1:1000), HO-1 (1:2000), GAPDH (1:1000), PI3K (1:1000), p-PI3K (1:1000), Akt (1:1000), p-Akt (1:2000). They were exposed to corresponding secondary antibodies for 1h at room temperature. Captured images were analyzed using Image J.

2.2.6 Statistical Analysis

The results were defined as the arithmetic mean ± standard deviation. Analysis was conducted using one-way ANOVA and Tukey's test, employing Graphpad Prism 9. $P < 0.05$ was considered to hold statistical significance, while $P < 0.01$ indicated a notable disparity.

3. Results

3.1 Drug-disease Interaction Network Analysis

A total of 11,217 disease-associated genes were delineated. A convergence of 182 genes was observed (Fig. 1A), indicating that it may be involved in the pathway relevant to HCC progression and exert significant regulatory effects. The result of Gene Ontology (GO) analysis (Fig. 1B) revealed that the predominant biological process (BP) enrichments included cellular reactive oxygen species responses, and oxidative stress; the predominant cellular component (CC) enrichments included secretory granule fluid compartment, etc. the dominant molecular function (MF) enrichments included phosphoprotein binding ability, and DNA binding, etc. Protein-protein interacting (PPI) network (Fig. 1C) indicated that AKT1, PIK3R1, NFE2L2, and HMOX1 might be key factors with enhanced therapeutic significance in utilizing Tan IIA for HCC therapy.

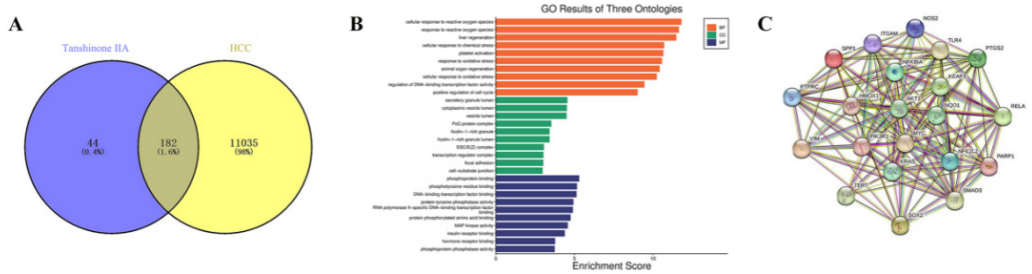


Fig 1. Drug-disease interaction network analysis. (A) Venn diagram showing cross genes of Tan IIA with HCC. (B) Histogram of GO analysis for Tan IIA. (C) PPI network diagram of potential targets of Tan IIA for hepatocellular carcinoma.

3.2 Evaluation of Antioxidant Activity in Huh7 Cells Induced By H_2O_2

Cell survival surpasses 80% post exposure to specific reagents, the chemical agent in question does not inflict any toxicity [9]. low doses of Tan IIA (0.25 and 0.5 μM) did not reduce cell survival (Fig. 2A). Tan IIA extracts showed little cytotoxicity when Tan IIA were maintained at 1, 2, and 4 μM, and thus 1, 2, and 4 μM concentrations of Tan IIA were selected for subsequent assays.

Excessive H_2O_2 can cause oxidative stress, therefore, H_2O_2 can be used to induce cellular oxidative damage models [10]. Upon increasing the concentration of H_2O_2, Huh7 cells displayed a concentration-dependent decrease in cellular viability (Fig. 2B). Therefore, 200 μM H_2O_2-induced Huh7 cells were chosen to establish the oxidative damage model.

The viability of Huh7 cells in the H_2O_2-induced cell model group remarkably diminished by 35.89% (Fig. 2C). As the concentration of Tan IIA was escalated from 1 μM to 4 μM, it exhibited a substantial protective effect, demonstrating a clear dose-dependent response. The findings suggest that Tan IIA plays a defensive part in preventing oxidative harm in Huh7 cells.

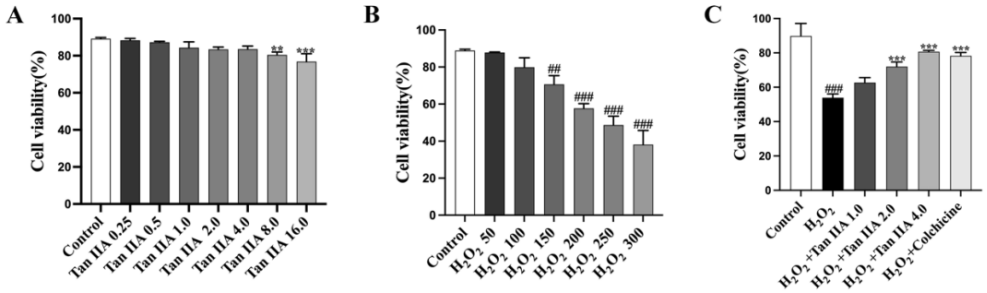


Fig 2. Impact of Tan IIA on H_2O_2-induced viability of Huh7 cells. (A) Consequence of diverse concentrations of Tan IIA on Huh7 cell viability. (B) Consequences of varied concentrations of H_2O_2 on Huh7 cell viability. (C) Tan IIA's role in preventing oxidative harm in Huh7 cells triggered by H_2O_2. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the model group.

3.3 Effect of Tan IIA on H_2O_2-Induced Antioxidant Enzyme Activities in Huh7 Cells

There was a significant decline in H_2O_2-induced SOD as well as GSH-Px activities in Huh7 cells in comparison to control group (Fig. 3). Activities of SOD alongside GSH-Px were augmented by diverse concentrations of Tan IIA, particularly at the elevated concentration of 4 μM. The intracellular MDA level of H_2O_2-treated Huh7 cells increased by 173%, while decreased significantly after treatment with Tan IIA. In conclusion, the H_2O_2-induced antioxidant defense system of Huh7 cells responded positively to oxidative stress.

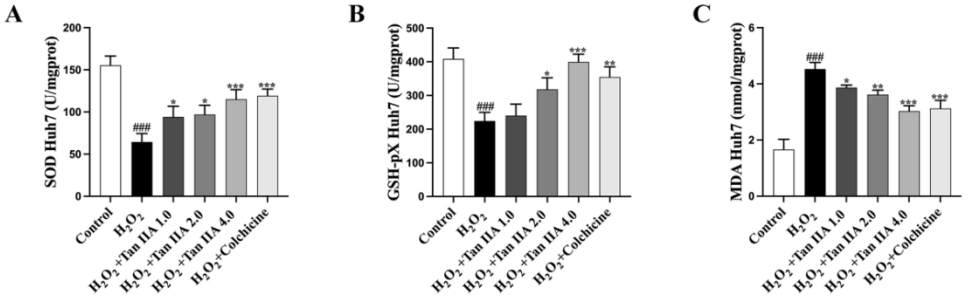


Fig 3. Results of varying dosages of Tan IIA on H_2O_2-elicited antioxidants enzyme levels. (A) SOD, (B) GSH-Px and (C) MDA in the Huh7 cell line. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the model group.

3.4 Impact of Tan IIA on the Levels of PI3K/Akt And Nrf2/HO-1 Pathway Proteins in Huh7 Cells Induced By H_2O_2

The possible impact of Tan IIA on oxidative stress was assessed by measuring p-PI3K, p-Akt, Nrf2, and HO-1 proteins through Western blot techniques (Fig. 4), and quantities of Nrf2 and HO-1

proteins were measured using immunofluorescence techniques (Fig. 5). H_2O_2-induced cell population demonstrated a marked increase upon observation of p-PI3K and p-Akt expression, treatment with Tan IIA significantly reversed it. In the realm of Nrf2/HO-1 signaling cascade, expression of Nrf2 alongside HO-1 proteins were considerably diminished exposed to H_2O_2 alone; however, the administration of Tan IIA augmented the expression of these proteins. Quantitation analysis from immunofluorescence revealed that the nuclear import of Nrf2 and HO-1 proteins was curtailed in H_2O_2-treated group, yet varying concentrations of Tan IIA effectively promoted concentration of the protein Nrf2 within the nucleus, coupled with an increase in HO-1 levels.

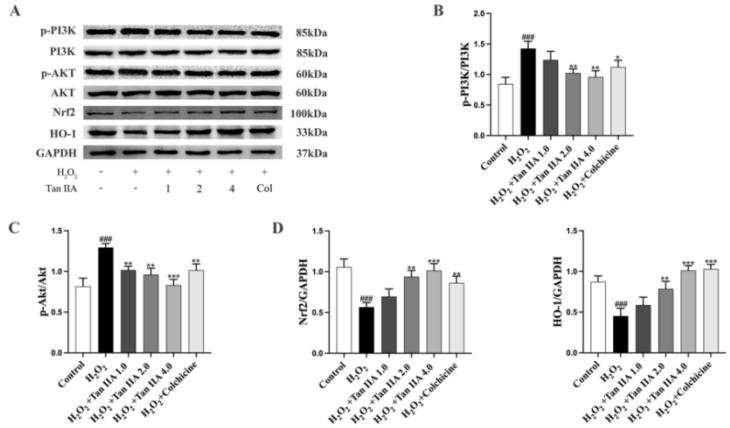


Fig 4. Tan IIA controls PI3K/Akt and Nrf2/HO-1 pathways following H_2O_2 stimulation of Huh7. (A), (B), (C), (D) and (E) Analysis via immunoblotting and the comparative protein quantities of p-PI3K, p-Akt, Nrf2, and HO-1. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the model group.

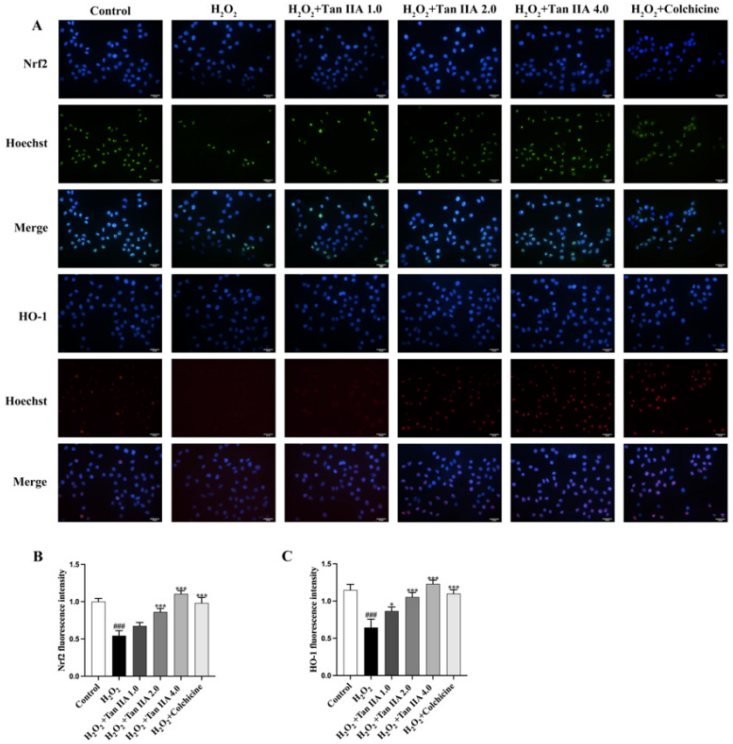


Fig 5. The immunofluorescence characteristics of Huh7 cells. (A), (B), (C) Analysis of Nrf2 and HO-1's expression and positioning through immunofluorescence, alongside the comparative intensity of immunological fluorescence for Nrf2 and HO-1 (magnification × 400). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the control group and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the model group.

4. Discussion

The oxidative stress manifests itself as a severe dysregulation of the energy metabolic balance between antioxidant defense systems and free radicals ROS [11]. Liver diseases is often accompanied by excessive ROS production, which in turn triggers increased oxidative stress. Evaluations of MDA levels are utilized as a fundamental indicator of the degree of oxidative stress [12]. Moreover, SOD is pivotal in the neutralization of superoxide, which is deemed as one of the critical indicators of the metabolic status of free radicals within the body [13]. GSH is a non-enzymatic free radical scavenger, can effectively eliminate oxygen ions and other free radicals, prevent the over-oxidation of fats, and a reduction in the concentration of this substance serves as a conspicuous indicator that the liver is progressively evolving towards necrosis [14]. If GSH continues to decrease, the antioxidant defense system will not function properly, leading to the accumulation of ROS. Both SOD and GSH-Px are considered as core components of the oxidative stress antioxidant defense system [15]. In this investigation, we demonstrated that under the influence of Tan IIA, the content of MDA significantly diminished, whilst the elevation of the levels of SOD and GSH-Px suggested a gradual increase, indicating that the active constituents incorporated in Tan IIA possess the potency to mitigating oxidative stress, and can augment the activity levels of the aforementioned antioxidant factors to counteract the damage inflicted by ROS.

Firstly, it is significant that Nrf2, as a paramount regulatory protein for the oxidative stress response, whose activity is tuned by Keap1 and controls the expression of various antioxidant proteins. The Nrf2 signaling cascade serves as an indispensable hub for bolstering the resilience of the antioxidant response mechanism, considerably intensifying the impact of intrinsic antioxidant defense mechanisms [16]. However, abnormal changes in Nrf2 signaling can lead to increased oxidative stress damage and disruption of physiological redox homeostasis [17]. In physiological conditions, Nrf2 interacts with Keap1, but during oxidative stress, it separates from Keap1 and moves into the nucleus, associates with the ARE region and fulfills a pivotal regulatory function in the induction of numerous antioxidant-related genes, such as HO-1, SOD, GSH, GSH-Px, and NQO1 etc. In particular, HO-1 possesses a wide range of functions such as resistance to apoptosis, inhibition of inflammatory responses, and antioxidant damage [18]. Stimulation of the Nrf2 signaling cascade notably augments protein abundance of antioxidant genes, encompassing HO-1 alongside NQO-1 [19]. We analyzed and assessed the Nrf2-pathway associated protein expression profile alongside its vital downstream antioxidant determinant, HO-1. Practical observations illustrated that the quantitative analysis of Nrf2 and HO-1 protein expressions in H_2O_2-induced Huh7 cells were notably suppressed. Post-treatment with Tan IIA, these indicators surged, indicating that Tan IIA possesses the potential to mitigate oxidative stress damage and bolster resistance to oxidative stress. Its primary mechanism of action is to avert further exacerbation of liver injury by bolstering the expression of Nrf2 and HO-1 signaling cascade.

PI3K/Akt signaling cascade can significantly influence the proliferation and metastasis of hepatocellular carcinoma [20]. Akt emerges as a pivotal effector exerting the functions of PI3K, among which AKT1 has a promotional effect on liver tumor formation, proliferation, and metastasis. Inhibition of this pathway effectively suppresses HCC metastasis. Both PI3K/Akt and Nrf2 signaling cascade demonstrate protective responses against oxidative stress, phosphorylated Akt triggers downstream antioxidant enzymes such as HO-1 [21]. Inhibition of PI3K/Akt pathway, coupled with an increase in Nrf2/HO-1 pathway, was noted to impede the advancement of HCC [22]. Our results showed that Huh7 cells treated with Tan IIA, PI3K and Akt protein expression was unaffected, but phosphorylated PI3K and Akt expression was significantly reduced. We hypothesized that the therapeutic and protective effects of Tan IIA might be achieved by inhibiting PI3K/Akt and activating Nrf2/HO-1 signaling cascade in reaction to oxidative stress in HCC, to ameliorate progression of HCC. Simultaneously, we identified that Tan IIA demonstrates multi-targeting capabilities through the network pharmacology analysis.

In conclusion, Tan IIA can elicit antioxidant effects via various mechanisms and targets in order to attain certain prevention and mitigation of HCC, potentially linked to suppression of PI3K/Akt pathway and upregulation of Nrf2/HO-1 signaling cascade.

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