Biomedicine & Pharmacotherapy 85 (2017) 611–619

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Txn1, Ctsd and Cdk4 are key proteins of combination therapy with taurine, epigallocatechin gallate and genistein against liver fibrosis in rats

Wen Cao[a,1], Yan Li[b,1], Min Li[c], Xuerong Zhang[c], Ming Liao[c,*]

[a] Department of Pharmacology, Guangxi Medical University, Nanning, China
[b] Guangxi University Library, Guangxi University, Nanning, China
[c] Medical Scientific Research Centre, Guangxi Medical University, Key Laboratory of High-Incidence-Tumor Prevention & Treatment (Guangxi Medical University), Ministry of Education, China

ARTICLE INFO

Article history:
Received 27 September 2016
Received in revised form 11 November 2016
Accepted 16 November 2016

Keywords:
Epigallocatechin gallate
Genistein
iTRAQ
Liver fibrosis
Proteomic
Taurine

ABSTRACT

The anti-fibrotic mechanism of combination therapy with taurine, epigallocatechin gallate and genistein was studied from the perspective of serum proteomics in our previous work. In order to further investigate and systematically analyse other possible therapeutic mechanism of combination therapy against liver fibrosis, isobaric tags for relative and absolute quantification (iTRAQ) proteomic analysis was applied to study the protein profile changes in liver tissue of carbon tetrachloride-induced liver fibrosis rats after combination therapy. A total of 115 differentially expressed proteins containing 84 up-regulated and 31 down-regulated proteins in response to combination therapy were identified. Three differentially expressed proteins (Txn1, Ctsd and Cdk4) involved in antioxidant defense system and the activation and proliferation of hepatic stellate cell were selected for further validation by western blot and real-time PCR analysis. Our study highlight the importance of differentially expressed proteins Txn1, Ctsd and Cdk4 against liver fibrosis, which may provide a more precise and comprehensive perspective for clarifying the roles of combination therapy as a potential agent for treatment of liver fibrosis.

1. Introduction

Liver fibrosis is caused by various chronic liver insults and represents a common and complex clinical challenge worldwide. During fibrogenesis, hepatic stellate cell (HSC) activation and collagen deposition stimulate the accumulation of extracellular matrix (ECM), which may destroy normal architecture of liver tissue [1,2]. Untreated fibrosis may lead to irreversible cirrhosis and organ malfunction [3]. Thus, the development of anti-fibrotic therapies is imperative.

Taurine, first extracted from bezoar, is a kind of animal sources of traditional Chinese medicine (TCM), and possesses a protective effect on the heart, liver, and eye. Epigallocatechin gallate (EGCG) is the most effective active constituent of tea polyphenols extracted from green tea. It has various pharmacological effects, such as antioxidant, anti-inflammatory, and anti-tumor. Genistein, an isoflavone derived from leguminous plants *Parochetus communis*, was found to inhibit the proliferation and activation of HSC. Our previous studies suggest that combination therapy with taurine, EGCG and genistein is more effective for the treatment of liver fibrosis than monotherapy [4,5]. Furthermore, through serum proteomics technology, we found that combination therapy could effectively prevent liver fibrosis mainly via regulating antioxidant defense, glycolysis and coagulation pathway [6]. However, it is still not comprehensive to study the anti-fibrotic mechanism of combination therapy only from the perspective of serum proteomics, since the liver is the main target tissue in liver fibrosis.

In this study, we applied isobaric tags for relative and absolute quantification (iTRAQ) combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS) to study changes of protein profile in liver tissue of CCl_4-induced liver fibrosis rats after combination therapy. The aim of this study is to further investigate and systematically analyse other possible therapeutic mechanism of combination therapy against liver fibrosis.

* Corresponding author at: Guangxi Medical University, 22 Shuangyong Road, Nanning, 530021, Guangxi, China.
E-mail address: lminggx@163.com (M. Liao).
[1] These two authors contributed equally.

http://dx.doi.org/10.1016/j.biopha.2016.11.071
0753-3322/

2. Materials and methods

2.1. Animals and diet

The experiments were performed on male Sprague-Dawley rats (220–250 g, SPF), purchased from Experimental Animal Center of Guangxi Medical University (Guangxi, China). The research was performed approved by the institutional ethical committee of Guangxi Medical University. Animals were housed under standard conditions (temperature: 25 ± 2 °C, relative humidity: 60 ± 10%, lighting cycle: 12 h light–dark cycle), fed with free access to food and water, and acclimated for a week.

2.2. Experimental design

According to the procedure of our previous study [6], animals were randomly divided in three groups and respectively treated with: (i) normal control group (n = 6, saline only), (ii) CCl_4 model group (n = 6, 2 ml/kg CCl_4 twice a week for 8 weeks and saline once a day for 6 weeks) and (iii) combination therapy group (n = 7, 2 ml/kg CCl_4 twice a week for 8 weeks and taurine 100 mg/kg+ EGCG 15 mg/kg + genistein 10 mg/kg once a day for 6 weeks by intragastric administration) (taurine, Chengdu Kelong Chemical Reagent Factory, Sichuan, China; EGCG, Leshan Yujia Tea Science and Technology Development Co., Ltd., Sichuan, China; genistein, Meryer Chemical Technology Co., Ltd., Shanghai, China). All animals were anesthetized and sacrificed after the treatment, and liver samples were collected.

2.3. Liver histological analysis

As described previously [6], processed by conventional histological procedures, a piece of right liver lope was fixed in 10% formalin, embedded in paraffin, sliced into 5 μm section. The sections were stained with haematoxylin-eosin (HE) staining and observed by DMR +Q550 pathological image analyzer at × 40 magnification (Leica Microsystems, Wetzlar, Germany). The fibrosis stages of the liver sections were scored in accordance with the histological METAVIR classification [7] in blind manner.

2.4. Protein extraction and quantization

Equal amounts of liver tissues from each rat of CCl_4 model group and combination therapy group were pooled to 2 distinct pools of 1 g each. Samples were grinded to power and dissolved in lysis buffer at 30 °C for 1 h. Centrifuge the solution by 15,000 *g* for 15 min and collect the supernatant. The supernatant was the extracted protein solution. The concentrations of the protein extracts were determined by the Bradford method.

2.5. iTRAQ analysis

Protein samples were reduced, blocked and digested with Trypsin Gold (Promega, Madison, WI, USA) and labeled using an iTRAQ Reagent Multiplex Kit (Applied Biosystems) according to the manufacturer's protocol. The liver samples of CCl_4 model and combination therapy group were labeled with 121 and 119 Da, respectively. Then, all samples were pooled and purified using a Poly-SEA strong cation exchange chromatography (SCX) column (2.0 × 150 mm, 5 um, Michrom) with Agilent 1200 HPLC system. The samples were eluted with SCX buffer B (500 mM FA, in 20% ACN) 0%–5% over 5 min, 5%–50% over 35 min and 50%–80% over 10 min on the Eksigent nanoLC-Ultra™ 2D System (AB SCIEX). The fractions were analyzed using a TripleTOF 5600 System (AB SCIEX, USA) with a Nanospray III source (AB SCIEX, USA) and a pulled quartz tip as the emitter (New Objectives, USA). Data were obtained using an ion spray voltage of 2.5 kV and an interface heater temperature of 150 °C. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. The iTRAQ analysis was performed as three technical replicates.

2.6. Data processing and bioinformatics analysis

Data were processed with Protein Pilot Software v. 4.5 (AB SCIEX, USA) against *Rattus* database using the Paragon algorithm. Protein was considered to be identified if at least two unique peptides match with 95% or greater confidence. Proteins quantified with a fold change of an average iTRAQ ratio >1.50 or <0.67 were identified to be significantly differentially expressed. The functional classifications and pathway annotation of the differentially expressed proteins were performed using DAVID Functional Annotation Tool. Protein interaction network was analyzed by STRING.

2.7. Western blot

Western blot validations were performed on Thioredoxin (Txn1), Cathepsin D (Ctsd), Cyclin-dependent kinase 4 (Cdk4). As described previously [6], liver tissues were lysed in RIPA lysis buffer for 10 min. Centrifuge the lysates at 15,000 *g* for 15 min and collect the supernatants. The concentrations of the protein extracts were also determined by the Bradford method. Proteins were separated with 10% SDS-PAGE and transferred onto PVDF

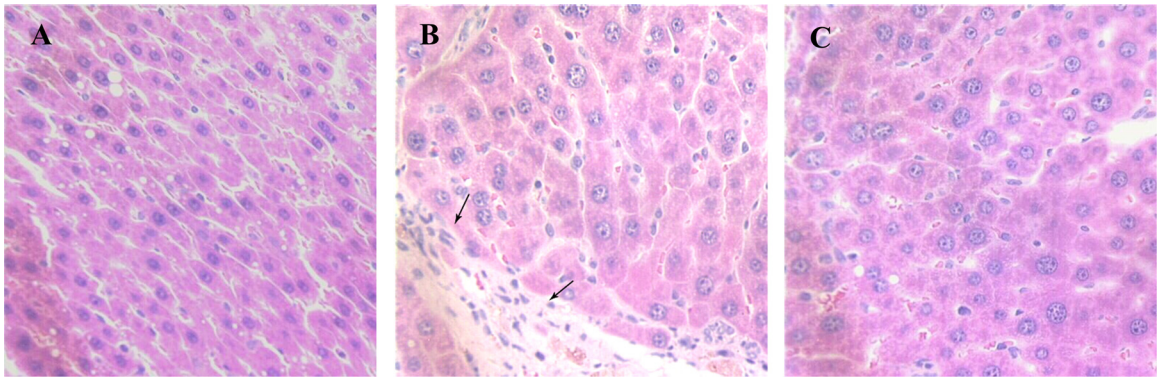

Fig. 1. Pathological histology analysis in liver tissues of normal control (A), CCl_4 model (B) and combination therapy group (C) by HE staining (the histological changes were marked by black arrows), magnification × 40.