Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2016, **14**(4): 0255–0264
Chinese Journal of Natural Medicines

Protective effects of curcumin against liver fibrosis through modulating DNA methylation

WU Peng [1, 2], HUANG Rui [1], XIONG Ya-Li [1], WU Chao [3*]

[1] *The First Clinical Medical School, Nanjing University of Traditional Chinese Medicine, Nanjing 210023, China;*
[2] *Department of Medical Nursing, Jiangsu Jiankang Vocational College, Nanjing 210029, China;*
[3] *Drum Tower Clinical School of Integrated Chinese and Western Medicines, Nanjing University of Traditional Chinese Medicine, Nanjing 210008, China*

Available online 20 Apr., 2016

[ABSTRACT] Recent research has demonstrated that advanced liver fibrosis in patients could be reversed, but no approved agents are available for the treatment and prevention of liver fibrosis in humans. Curcumin (CUR) is the principal curcuminoid of turmeric. Inhibitory effects of CUR and its underlying mechanisms in liver fibrogenesis have been explored. In the present study, we hypothesized that epigenetic mechanisms contribute to the protective effects of CUR against liver fibrosis. We used CCl_4-induced liver injury in BALB/c mice and the rat hepatic stellate cell line HSC-T6 as experimental models. Genomic DNA methylation was analyzed by MeDIP-chip and verified by real-time PCR on MeDIP-enriched DNA. The mRNA and protein expressions of DNMT1, α-SMA, and Col1α1 were determined by real-time PCR and Western blotting, respectively. The methylation statuses of FGFR3, FZD10, Gpx4, and Hoxd3 were further confirmed by quantitative methylation-specific PCR (qMSP). Our results showed that CUR treatment reversed liver injury *in vivo* and *in vitro*, possibly through down regulation of DNMT1, α-SMA, and Col1α1 and by demethylation of the key genes. In conclusion, aberrant methylation is closely associated with liver fibrosis and CUR treatment may reverse liver fibrosis by epigenetic mechanisms.

[KEY WORDS] Liver fibrosis; Curcumin; DNA methylation; Therapy

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2016)04-0255-10

Introduction

Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) proteins, including collagen, in the extracellular spaces. It may occur in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis and sometimes hepatocellular carcinoma (HCC), leading to liver failure, portal hypertension, and even death [1]. Activated hepatic stellate cells (HSCs) are the major source of collagen products, causing an imbalance between the formation and degradation of ECM in the liver tissues [2]. Myofibroblasts of bone marrow origin and portal fibroblasts also contribute to collagen production in the injured liver [3]. These cells are activated by fibrogenic cytokines such as TGF-β1, angiotensin II and leptin and regulated by proinflammatory cytokines such as NF-κB, TNF-α and IL-6 [4-5]. Recent research has demonstrated that advanced liver fibrosis could be reversed in patients, encouraging the development of antifibrotic drugs [6]. Potential antifibrotic therapies are aimed at inhibiting activation of fibrogenic cells, inducing apoptosis of activated HSCs, and/or preventing deposition of ECM proteins. Currently, no approved agents are available for the treatment and prevention of liver fibrosis in humans.

Chinese traditional medicine has achieved successful results in treating hepatic diseases for centuries [7]. The curcuminoids are natural phenols responsible for the yellow color of turmeric and curcumin (CUR) is the principal curcuminoid. Turmeric has been used as a component of Chinese traditional medicine for thousands of years. In the latter half of the 20th century, CUR was identified as the agent responsible for most of the biological activity of

[Received on] 10-Oct.-2015
[Research funding] The work was supported by Jiangsu Provincial Bureau of Traditional Chinese Medicine (No. LZ13241), National Natural Science Foundation of China (No. 81470093), Jiangsu Provincial Outstanding Medical Academic Leader Program (No. LJ201154), and Jiangsu Provincial Clinical Medicine and Technology Special Program (No. BL2012034).
[*Corresponding author] Tel: +86-25-83304616, Fax: +86-25-83304616, E-mail: dr.wu@nju.edu.cn
These authors have no conflict of interest to declare.

turmeric [8]. In both *in vitro* and animal studies, CUR showes antitumor [9], antioxidant [10], and anti-inflammatory properties [11]. Inhibitory effects of CUR and its underlying mechanisms in liver fibrogenesis have also been explored [12]. Previous studies have shown that CUR targets multiple pathways to halt HSC activation [13]. Recent studies have highlighted regulatory effects of epigenetic modifications at the level of gene expression [14]. Various types of modifications, including DNA methylation, histone modification and chromatin structure changes, are of potential relevance to fibrogenic processes [15]. Several studies have reported that CUR is an epigenetic regulator in neurological disorders, inflammation and diabetes, in addition to cancers [16]. The epigenetic effects of CUR include the following [17]: (1) inhibition of DNA methyltransferases (DNMTs), well defined in recent studies showing its effects as a DNA hypo-methylating agent; (2) regulation of histone modification via regulation of histone acetyltransferases (HATs) and histone deacetylases (HDACs); and (3) regulation of micro RNAs (miRNA). Thus, we hypothesized that epigenetic mechanisms contribute to the protective effects of CUR in liver fibrosis.

Materials and Methods

Liver injury model

Thirty male BALB/c mice (5 weeks old) were purchased from the Academy of Military Medical Science (Beijing, China) and housed in the Comparative Medicine Department of Gulou Hospital (Nanjing, China). The mice were housed under controlled temperature and humidity, with a 12-h/12-h light/dark cycle and sterile food and water provided *ad libitum*. The animal studies were carried out in strict accordance with the guidelines for the Care and Use of Laboratory Animals of Nanjing University of Chinese Medicine, Nanjing, China. All efforts were made to minimize pain and suffering. Liver fibrosis (FM group) was generated with CCl_4 (CCl_4 : olive oil, 1 : 1 (*V* : *V*) at 1 mL per kg body weight by intraperitoneal injection twice weekly for 8 weeks) as previously described [18]. Vehicle control animals (FC group) were injected intraperitoneally with 1 mL of olive oil per kg body weight at the same time intervals. CUR treatment (FJ group) involved oral administration of 50, 100, and 200 $mg \cdot kg^{-1}$ of CUR three times a week for 8 weeks. At 24 h after the last CCl_4 injection, the mice were sacrificed and liver tissue and blood samples were harvested for further analysis. Livers were fixed with 10% formalin solution and embedded in paraffin, and 4-μm-thick sections were prepared, deparaffinized with xylene and rehydrated in water through a graded ethanol series. Liver sections were stained with hematoxylin and eosin (H&E) or Masson staining. Serum ALT and AST levels were analyzed by an automatic biochemical analyzer (Beckman Coulter AU680, Boulevard, CA, USA).

MeDIP-on-chip

Genomic DNA was extracted from liver samples, using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), and sonicated at about 200–1 000 bp with a Bioruptor sonicator (Diagenode, Denville, NJ, USA). Equal amounts of DNA from each mouse within the group (six per group) were processed. About 1 μg of DNA was sonicated and then subjected to immunoprecipitation using a mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Belgium, NJ, USA). DNA was heat-denatured at 94 °C for 10 min, rapidly cooled on ice and immunoprecipitated with 1 μL of primary antibody overnight at 4 °C with rocking agitation in 400 μL of immunoprecipitation buffer (0.5% BSA in PBS). To recover the immunoprecipitated DNA fragments, 200 μL of anti-mouse IgG magnetic beads were added and incubated for an additional 2 h at 4 °C with agitation. After immuno-precipitation, a total of five washes were performed with ice-cold immunoprecipitation buffer. Washed beads were re-suspended in TE buffer with 0.25% SDS and 0.25 $mg \cdot mL^{-1}$ proteinase K for 2 h at 65 °C, then allowed to cool to room temperature. MeDIP DNA was purified using Qiagen MinElute columns (Qiagen). The MeDIP-enriched DNA was amplified using a WGA kit (GenomePlex® Complete Whole Genome Amplification (WGA2) kit) from Sigma-Aldrich (St. Louis, MO, USA). The amplified DNA samples were then purified with a QIAquick PCR purification kit (Qiagen). For DNA labeling, the NimbleGen Dual-Color DNA Labeling Kit was used according to the manufacturer's instructions, as detailed in the NimbleGen MeDIP-chip protocol (Roche-NimbleGen Systems, Inc., Madison, WI, USA). An aliquot of 1 μg of DNA from each sample was incubated for 10 min at 98 °C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (Input sample). Then, 100 pmol of deoxynucleoside triphosphates and 100 U of Klenow fragment (New England Biolabs, Beverly, MA, USA) were added and the mixture was incubated at 37 °C for 2 h. The reaction was stopped by adding 0.1 volume 0.5 $mol \cdot L^{-1}$ of EDTA and the labeled DNA was purified by precipitation in isopropanol: ethanol (1 : 1). Microarrays were hybridized at 42 °C for 16 to 20 h with Cy3/5 labeled DNA in Nimblegen hybridization buffer/hybridization component A in a hybridization chamber (Roche-NimbleGen). Following hybridization, washing was performed using the Nimblegen Wash Buffer kit. For array hybridization, Roche-NimbleGen's Mouse Promoter plus CpG island array was used, a 3 × 720 k format array design containing 15 980 annotated CpG islands as well as 20 404 promoters (from about –2 960 bp to 740 bp of the TSSs) was used to totally cover approximately 720 000 probes [19].

Microarray data analysis

From the normalized log2-ratio data, a sliding-window (750 bp) peak-finding algorithm provided by NimbleScan v2.5 (Roche-NimbleGen) was applied to analyze the MeDIP-chip data. A one-sided Kolmogorov-Smirnov (KS) test was used to determine whether the probes were drawn from a significantly more positive distribution of intensity log2-ratios than those in the rest of the array. Each probe received a –log10 *P*-value score from the windowed KS test around that probe. If several adjacent probes rose significantly