Journal of Proteomics 177 (2018) 40–47

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Transcriptome analysis to assess the cholestatic hepatotoxicity induced by Polygoni Multiflori Radix: Up-regulation of key enzymes of cholesterol and bile acid biosynthesis

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ARTICLE INFO

Keywords:
Polygoni Multiflori Radix
Cholestatic liver injury
Transcriptome analysis
Cholesterol biosynthesis
Bile acid biosynthesis

ABSTRACT

Polygoni Multiflori Radix (PMR) has been commonly used as a tonic in China for centuries. However, PMR-associated hepatotoxicity is becoming a safety issue. Cholestasis often occurs in PMR-induced hepatotoxicity in clinical medicine, but the exact mechanism is not completely understood. An RNA-Seq method was employed, in the present study, to explore the molecular mechanism of cholestatic liver injury induced by PMR, characterized by the hepatic transcriptional response in rats exposed to 1 and 20 g/kg PMR for 90 days. Pathological changes seen in rat livers exposed to PMR included increased bile ducts in portal areas and biliary epithelial cell hyperplasia, which were accompanied by the elevation of serum biochemistries. Dose-dependent increases in the expression of 14 transcripts encoding enzymes involved in the cholesterol biosynthetic pathway were identified. Furthermore, cholesterol 7-alpha hydroxylase (Cyp7a1), a rate-limiting enzyme in the synthesis of bile acids (BAs) from cholesterol, was found to be upregulated by PMR treatment. Protein analysis by western blot suggested that expression of 3-hydroxy-3-methylglutaryl CoA reductase (Hmgcr) and Cyp7a1 were increased in a dose-dependent manner. Collectively, the present study demonstrates that PMR upregulates key enzymes for biosynthesis of cholesterol and BA, which poses the risk of cholestatic liver injury.

Significance: To the best of our knowledge, this is the first transcriptome analysis to highlight the main molecular changes occurring in rats chronic exposed to PMR. We have identified 39 specific differentially expressed genes (DEGs) that were present in various comparisons. A total of 14 of these altered gene transcripts were associated with cholesterol biosynthesis. Another factor of great importance in our opinion seemed to be the enhancement of bile acid (BA) biosynthesis, which were closely linked to cholesterol biosynthesis or metabolism. Our findings suggested that the disturbance on balance of BA formation and elimination might lead to a BA overload in hepatocytes, thereby resulting in liver injury.

1. Introduction

Polygoni Multiflori Radix (PMR, Chinese name: Heshouwu) is a commonly consumed traditional Chinese herbal medicine that originates from the dried root of *Polygonum multiflorum* Thunb (family Polygonaceae). As one of the four most precious herbs in Chinese medicine, PMR earns its reputation owing to its powerful tonifying effects, and therefore, appears in many herbal prescriptions. Recently, with increased public health awareness, PMR and its preparations are widely used in over the counter drugs, dietary supplements, and even in daily diets. The toxicity of this plant was first described in *Bencaohuiyan*, a classic monograph of traditional Chinese medicine written during the Ming dynasty (1368–1644 CE), which described this plant to be of minimal toxicity to humans [1]. In 2006, a safety alert about PMR was posted on the MHRA (Medicines and Healthcare Products Regulatory Agency, UK) website after seven cases of liver injury were reported through the Yellow Card Scheme. This announcement also cited the cases of liver injury by taking a preparation containing PMR that occurred in Hong Kong, Australia, Italy and Holland [2]. Since then, PMR-induced liver injury has attracted worldwide attention. In Canada, British and Australia, restrictions were put forth to supervise and manage the use of PMR-containing products for clinical applications [3]. In China, a series of pharmacovigilance reports about PMR and PMR-containing preparations were announced by China Food and Drug Administration (CFDA) [4]. After 2010, the recommended PMR dose was reduced from 6–12 g to 3–6 g in the Chinese Pharmacopoeia for safety purpose [5,6].

Bile is a yellow/green aqueous solution of organic and inorganic

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https://doi.org/10.1016/j.jprot.2018.02.014
Received 22 August 2017; Received in revised form 29 January 2018; Accepted 8 February 2018
Available online 10 February 2018
1874-3919/

compounds synthesized in the pericentral hepatocytes of the liver, and is stored and concentrated in the gallbladder during the interdigestive state. Upon dietary intake, bile is released into the duodenum [7]. Bile acids (BAs), which are synthesized in the liver from cholesterol by a multi-enzyme process, make up approximately 50% of organic components in the bile. BAs play an essential role in digestion by emulsifying and solubilizing fats [7]. Nevertheless, BAs are also known to be cytotoxic to hepatocytes [8–10]; excessive accumulation of BAs in the liver can cause cholestasis, and when severe can progress to liver diseases [11,12]. A leading hypothesis for the development of cholestatic liver disease is the accumulation of BAs in hepatocytes, which can reach potentially cytotoxic levels, and subsequently lead to BA increases in serum [13–15]. In clinic, partial cases of PMR-induced liver damage presented with cholestatis [16], and the main symptom was mild to moderate jaundice (yellow-colored urine, eyes, and skin). Perturbations in the homeostasis of BAs had been proposed as a common and early event of PMR-induced liver injury [17]. Our previous study demonstrated that PMR-induced hepatotoxicity might be related to disorders of BA metabolism, and urine tauro-β-muricholic acid was identified as a noninvasive biomarker for clinical monitoring of hepatotoxicity [18]. However, the molecular mechanism linking the entatic state of BAs to cholestatic liver injury remains unclear.

Transcriptomics is now a robust technology capable of simultaneously quantifying tens of thousands of defined mRNA species in a miniaturized, automated format [19]. The combination of transcriptomics with general toxicity studies has emerged to predict the development and progression of drug-induced hepatotoxicity [20–22]. For example, based on the analysis of the results from transcriptomics, Lu et al. [23] reported that p53 signaling pathway induced by oxidative damage was the crucial step in aflatoxin B1-induced acute hepatotoxicity. Therefore, transcriptomic technology may serve as a potent tool to comprehensively contribute to the knowledge of underlying mechanisms of hepatotoxicity. In the present study, we determined liver transcriptomes in rats that were chronically exposed to different doses of PMR, using a sequencing platform for identifying differentially expressed genes (DEGs) associated with cholestatic liver injury. We hope that the systematic transcriptome analysis used in this study may facilitate better understanding of the molecular mechanisms underlying the cholestatic liver injury induced by PMR.

2. Materials and methods

2.1. Animals

Sprague-Dawley (SD) rats were purchased from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China). Rats used in the studies were males weighing 200–220 g, and were housed in the Pharmaceutical Animal Experimental Center of China Pharmaceutical University with natural light-dark cycles, temperature and humidity controlled environments, and ad libitum access to diet and water for one week before the experiments was performed. The animal experiments were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Department of Science and Technology of Jiangsu Province (License number: SYXK (SU) 2016-0011).

2.2. Reagents

Decoction pieces of PMR were purchased from Bozhou traditional Chinese medicine market (Anhui, China), the specimens were authenticated by Prof. Hui-Jun Li and deposited at the State Key Laboratory of Natural Medicines (China Pharmaceutical University). The PMR extract was prepared according to our previous work [24]. Briefly, PMR decoction pieces were extracted thrice under reflux each with 10 times of 75% ethanol for 3 h. The three extracts were combined and concentrated under reduced pressure, then lyophilized, the concentrations of the main constituents including emodin, physcion and 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycopyranoside in PMR extract were 0.16%, 0.13%, and 5.69%, respectively. The lyophilized powders were re-dissolved and dispersed in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution for intragastric administration. The polyclonal anti-mouse Cyp7a1 antibody, predicted to cross-react with the rat antigen, the anti-rat Hmgcr antibody and the anti-rat Bsep antibody were purchased from Abcam (Cambridge, USA). The anti-rat Mrp3 antibody was from Santa Cruz (Texas, USA).

2.3. Sample collection

Male SD rats were randomly divided into three groups (a control group and two treatment groups) by intragastric administration of a vehicle (1 mL/100 g) or a low and high PMR extract dose (1 and 20 g/kg) for 90 days. Blood was drawn via the post-ocular vein 24 h after the last PMR administration. Plasma was obtained by centrifugation at 4000 rpm for 15 min at 4 °C and stored at −80 °C before use. At the end of the experiment, rats were sacrificed by cervical dislocation, and livers were harvested between 9:00 AM and 12:00 AM to minimize variations in gene expression of the drug-metabolizing enzymes. Part of the liver was rapidly quenched in liquid nitrogen and then stored at −80 °C until transcriptome analysis or hepatic total bile acid (TBA) measurement was performed, and the remaining liver segments were fixed in 10% formalin for histopathological examination. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities, and TBA and total bilirubin (TBIL) concentrations were determined using an automatic blood biochemical analyzer (Beckman Coulter, USA). Liver function testing kits were supplied by Zhongda Hospital (Nanjing, China). Hepatic TBA were measured using TBAs assay kit purchased from Changchun Huili Biotech Co., Ltd.(Chang Chun, China).

2.4. RNA extraction

Total RNA was isolated from the liver tissue of three randomly selected rats, one from each group, with Trizol (Invitrogen, USA) following the manufacturer's protocol. Total RNA concentrations in each sample were measured using a NanoPhotometer (Implen, USA). The quality of RNA was assessed by running samples on a denaturing agarose gel and then visualizing two discrete 18S and 28S ribosomal RNA bands, for which the ratio of the intensity of 28S band to that of 18S band was > 1. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent, USA). Samples with RIN (RNA integrity number) values larger than 7 were used for further analysis.

2.5. cDNA library preparation

cDNA libraries from the total RNA samples were prepared using an RNA Library Prep Kit (Neb, USA) following manufacturer's recommendations. Sample mRNA was concentrated using magnetic oligo-dT beads and then cleaved into fragments, which served as templates for the synthesis of the first-strand of cDNA using random hexamers and reverse transcriptase. The second strand of cDNA was synthesized using dNTPs, RNase H, and DNA polymerase I. A suitable length of the cDNA fragments was selected using agarose gel electrophoresis and amplified by PCR to construct the final cDNA libraries for sequencing on the Illumina HiSeq 4000 platform (Illumina, USA).

2.6. RNA-Seq data analysis

After the sequencing platform generated sequencing images, raw sequence reads from nine samples were collected, and were subjected to quality-related processing, filtering, and digital normalization, after which de novo transcriptome assembly was conducted using Trinity (version r2013-02-25). Transcripts with a false discovery rate