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Serum metabonomics study of the hepatoprotective effect of amarogentin on CCl_4-induced liver fibrosis in mice by GC-TOF-MS analysis

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ABSTRACT

Amarogentin (AG) is a secoiridoid glycoside that is mainly extracted from the traditional Chinese medicine *Swertia* and *Gentiana*, which have been widely used in clinical practice to treat liver disease. However, the exact hepatoprotective mechanism of AG was still looking forward to further elucidation by far. In this study, C57BL/6 mice were divided into the following three groups: control, model and AG. Fibrosis was induced by CCl_4. Mice were orally treated with 100 mg/kg AG or with normal saline as a control. At the end of the experiment, the validity of the model and the hepatoprotective effects of AG were examined by histopathology and biochemical indicators. Metabonomics technology was further performed to systematically evaluate the endogenous metabolite profiles. Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) technology with pattern recognition analysis, including principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA), showed a clear separation of the model group and the control group, with the AG treatment group located much closer to the control group than the model group, which was consistent with the results of biochemical and histopathological assays. Moreover, nine potential biomarkers were identified to elucidate the drug mechanism of AG, which may be related to pathways of amino acid and fatty acid metabolism.

1. Introduction

Liver fibrosis is caused by a variety of chronic stimuli, including alcohol intake, immunological attack, drug abuse, cholestasis and hepatic viruses [1]. It is a pathological process characterized by the production and excessive deposition of extracellular matrix (ECM) [2]. Without effective therapy, liver injury may ultimately develop into hepatic failure or hepatocellular carcinoma (HCC), which ranks third in worldwide cancer mortality behind lung and gastric cancers [3]. Despite the tremendous advances in the field of modern medicine, efficient anti-fibrotic drugs with few side effects have not yet been developed. Currently, the treatment for hepatic fibrosis is limited to removal of the noxious agents and orthotropic liver transplantation for end-stage liver failure [4]. Thus, the search for new hepatoprotective drugs is an area of intense research activity. More recently, Chinese medicines and their formulas have aroused much interest, especially due to their superiority in treating liver fibrosis and their few side effects [5,6].

Amarogentin (AG) (Fig. 1) exhibits many biological effects, including anti-tumourigenic, anti-diabetic, and antioxidative properties [7]. Recent studies have addressed the prevention of liver carcinogenesis by AG through modulation of the G_1/S cell cycle checkpoint and induction of apoptosis [8]. Our previous study found the possible anti-fibrotic mechanisms by which AG regulates oxidative stress and the MAPK pathways [9]. However, novel approaches to identify the mechanisms are urgently needed to provide a comprehensive evaluation of the systemic clinical efficacy of AG. Rosenberg noted the urgent need to establish reliable, reproducible and non-invasive surrogate serum indicators to monitor fibrosis progression. Careful selection of research methods and proper data interpretation are vital [10]. Metabonomics is defined as "the quantitative measurement of the dynamic multiparametric responses of a living system to pathophysiological stimuli or

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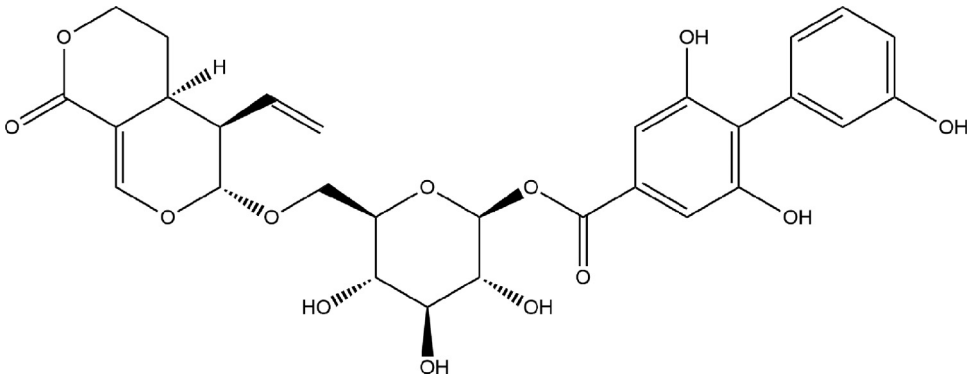

Fig. 1. Chemical structure of amarogentin (AG, chemical name: (4a*S*,5*R*,6*S*)-5-ethenyl-4,4a,5,6-tetrahydro-6-[[2-*O*-[(3,3′,5-trihydroxy[1,1′-biphenyl]-2-yl)carbonyl]-β-D-glucopyranosyl]oxy]-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one).

genetic modification". It is one of the major emerging fields of systems biology and provides a powerful platform for simultaneously monitoring endogenous metabolite levels in biological fluid samples combined with a variety of pattern-analysis methods to reveal potential biomarkers of interest and identify related metabolic pathways [11]. Successful applications of metabonomics have been used in hepatitis research, and metabonomics is a feasible and powerful tool for the identification and quantitation of biomarkers [12,13]. A number of analytical tools have been employed for metabonomic analysis, including proton nuclear magnetic resonance imaging (NMR), ultra-performance liquid chromatography with mass spectrometry (UPLC–MS), capillary electrophoresis with mass spectrometry (CE-MS) and gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) [14]. Among these analytical methods, GC-TOF-MS provides powerful separation efficiency and detection sensitivity, and it has become a useful analytical tool in the research field of metabonomics [15].

In this article, GC-TOF-MS-based metabonomics was used to investigate the effect of AG on metabolic profiles of mice with CCl_4-induced liver fibrosis. We used this approach to explore the possible anti-fibrotic mechanism of AG from the perspective of influential metabolic pathways by observing the change in serum metabolic profiles.

2. Materials and methods

2.1. Reagents and chemicals

Amarogentin ($C_{29}H_{30}O_{13}$, CAS#: 21018-84-8, MW 586.54, HPLC >99%) was provided by Xi'an Day Natural Inc. (Xi'an, China), and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Regis Technologies, Inc (IL, USA). Methanol, chloroform, L-2-Chlorophenylalanine and pyridine were obtained from Shanghai Heng Bo Biological Technology Co. (Shanghai, China). CCl_4 was purchased from Tianjing Fuyv Chemical Reagent Co. Ltd. (Tianjing, China), and olive oil was purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Animals and treatment

Male C57BL/6 mice (6 weeks old; 20 ± 2 g) were purchased from the Experimental Animal Research Centre, the Fourth Military Medical University (Xi'an, China). All mice were housed in an Individually Ventilated Cage (IVC) and fed standard laboratory food and water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University (Approval No. 2015-1123-R). After one week of acclimation, the C57BL/6 mice were randomly divided into a control group, a model group and an AG group (n = 8). The model of liver fibrosis and the dose of AG were selected on the basis of previous studies [9]. Animals in the model and AG groups were hypodermic injected CCl_4 dissolved in olive oil (20% *v/v*, 6 mL/kg) twice per week for 7 consecutive weeks to establish the liver fibrosis model. The control group was injected with 6 mL/kg of olive oil at the same time point for comparison. After one week of CCl_4 administration, the control and model groups received equal quantities of normal saline; AG (100 mg/kg) was suspended in a 0.5% sodium carboxymethylcellulose solution and was orally administered once per day for six consecutive weeks.

At the end of the experimental period, the animals were anesthetized with an overdose of sodium pentobarbital. Blood specimens were collected via retro-orbital bleeding. After cruor at room temperature for 1 h, serum was collected after 3500 rpm centrifugation for 10 min and stored at −80 °C until analysis. The liver tissues were rapidly dissected and kept at −80 °C or fixed with 4% paraformaldehyde.

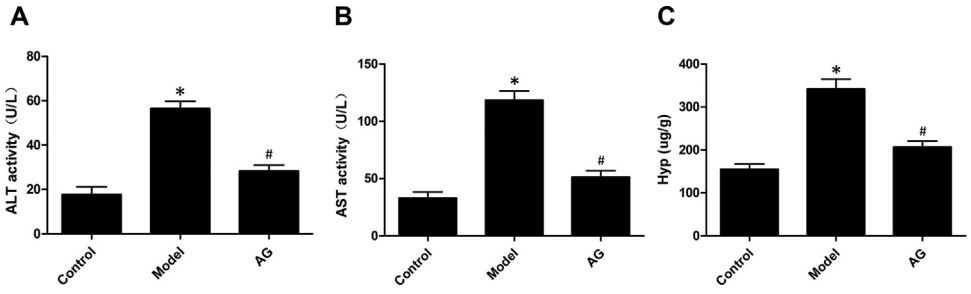

Fig. 2. ALT, AST and Hyp levels in liver tissues. Values are expressed as the means ± SD (n = 8) and were analysed using one-way ANOVA with Tukey's multiple comparisons test. * $P < 0.05$ versus the control group, # $P < 0.05$ versus the model group.