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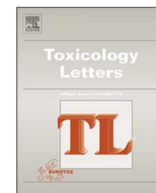

Serum microRNA-122-3p, microRNA-194-5p and microRNA-5099 are potential toxicological biomarkers for the hepatotoxicity induced by Airpotato yam

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ABSTRACT

Airpotato yam (the rhizome of *Dioscorea bulbifera* L.) is traditionally used to treat thyroid disease and various cancers in China. However, it was found to cause hepatotoxicity during clinical practice. This study aims to identify candidate serum microRNAs (miRNAs) as diagnostic biomarkers for the liver injury induced by Airpotato yam. The results of serum alanine/aspartate aminotransferase (ALT/AST) showed the remarkable hepatotoxicity induced by ethyl acetate fraction of Airpotato yam (EF) (450 mg/kg) and diosbulbin B (DB) (300 mg/kg) in mice. The results of miRNAs chip analysis showed that the expression of 28 and 37 serum miRNAs was obviously altered in EF- and DB-treated mice, respectively. Among these miRNAs, miRNA-122-3p, miR-194-3p and miR-5099 have passed the further validation in serum from both EF- and DB-treated mice. Moreover, the expression of miRNA-122-3p and miRNA-194-5p was significantly increased in EF (375 mg/kg)-treated mice with no significant elevation of serum ALT/AST activity. Only the expression of serum miRNA-5099 was not altered in the liver injury induced by acetaminophen (APAP), monocrotaline (MCT) or toosendanin (TSN). In conclusion, this study demonstrated that miR-122-3p and miRNA-194-5p were two sensitive biomarkers, and miR-5099 might be a specific biomarker for reflecting the liver injury induced by Airpotato yam.

1. Introduction

Drug-induced liver injury (DILI) is a type of liver injury caused by the drug itself or its metabolites (Schuster et al., 2005). According to the report of World Health Organization (WHO), DILI has risen to the fifth cause of death in the world. DILI has been reported to be associated with more than 1000 medications and is the most common cause of acute liver failure in the United States (Giordano et al., 2014). A prospective study of acute liver failure at 17 tertiary care centers in the United States demonstrated that approximately above 50% of acute liver failure in clinic was caused by drugs (Ostapowicz et al., 2002). With the wide acceptance of traditional Chinese medicines (TCMs) in the world, hepatotoxicity induced by herbal and dietary supplements has been a rising cause for DILI (Teschke et al., 2014). More and more cases of DILI due to the intake of TCMs were reported recently, providing new clinical challenges (Teschke et al., 2014). In China, 44.2% (total 337 cases) cases of liver injury were caused by Chinese herbal medicines from 2009 to 2012 (Ma et al., 2014a). In Singapore, 61.4% (total 57 cases) cases of liver injury were caused by TCMs from 2009 to 2014 (Teo et al., 2016). Even in United States and Europe, 2–11% of patients with DILI and 5–10% of patients with drug-induced acute liver failure were caused by herbal products (Bunchomtavakul and Reddy, 2013). Thus it can be seen that TCMs-induced liver injury has become a global issue that cannot be ignored.

ALT and AST are generally used by physicians to noninvasively reflect the existing liver injury. Due to the lack of specific biomarkers for DILI, DILI is still monitored and diagnosed based on these two classical serum biomarkers (Ozer et al., 2008). However, more and more reports demonstrated that the elevation of these two conventional biomarkers was not only related with liver injury (Nathwani et al.,

Abbreviations: miRNAs, microRNAs; ALT, alanine transaminase; AST, aspartate transaminase; EF, ethyl acetate fraction of Airpotato yam; DB, diosbulbin B; APAP, acetaminophen; MCT, monocrotaline; TSN, toosendanin; DILI, Drug-induced liver injury; WHO, World Health Organization; TCMs, traditional Chinese medicines; CHD, coronary heart disease; STEMI, ST-segment elevation myocardial infarction; CMC-Na, carboxymethylcellulose sodium; i.g., intragastric administration; H & E, haematoxylin and eosin; snRNA, small nuclear RNA; SEM, standard error of the mean; HCA, hierarchical clustering analysis [1]These two authors contributed equally to the work

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2005; Moon et al., 2014; Shen et al., 2015). A previous study showed that the highly increased serum ALT and AST activity was found in patients with skeletal muscle necrosis without the evidence of liver disease (Nathwani et al., 2005). High serum ALT and AST activity was also associated with coronary heart disease (CHD) and could be used as biochemical markers for predicting the severity of CHD (Shen et al., 2015). Additionally, the elevated serum ALT and AST activity was often observed in patients with acute ST-segment elevation myocardial infarction (STEMI) (Moon et al., 2014).

miRNAs are endogenously expressed small non-coding RNA molecule, containing about 22 nucleotides (Ambros, 2004). Accumulating studies demonstrate the critical roles of miRNAs in many human physiological and pathological processes, including cell proliferation, cell differentiation, apoptosis and cellular immune responses (Bartel, 2004, 2009). As for the stability of miRNA in blood and with the development of rapid and high-throughput miRNA chip detection technology, circulating miRNA has the huge potential to be served as a novel and minimally invasive biomarker for diagnosing human cancers and other diseases including DILI.

Airpotato yam is a traditionally used herbal medicine, but it was found to have toxicity during clinical practice. Our previous studies have shown that the main toxic target organ of Airpotato yam is liver, and its ingestion could lead to severe liver damage, including liver swelling, fatty degeneration and even death (Wang et al., 2010; Ma et al., 2014b). As described in our previous studies, the ethyl acetate extract (EF) isolated from Airpotato yam and diosbulbin B (DB), a main compound isolated from Airpotato yam, caused serious liver injury in experimental animals (Wang et al., 2010, 2011; Ma et al., 2014b). This study aims to find the candidate miRNA biomarkers with high sensitivity or specificity for reflecting the liver injury induced by Airpotato yam.

2. Materials and methods

2.1. Chemical compounds and reagents

Airpotato yam (the rhizome of *D. bulbifera*) was collected in Qingyang (Anhui Province) and authenticated by Prof. Shou-Jin Liu (Anhui College of Traditional Chinese Medicine, Anhui, China). The voucher specimens were deposited in the herbarium of Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. The preparation of ethyl acetate extract (EF) isolated from ethanol extract of Airpotato yam has already been reported in our previous published study, and the content of diosbulbin B (DB) in EF was 13.72% (Wang et al., 2012). DB (Purity > 98%) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). APAP was obtained from Sigma (St Louis, MO). MCT (Purity > 98%) was purchased from Nanjing GuangRun Biotechnology Co., Ltd (Nanjing, China). TSN (Purity > 98%) was obtained from Shanghai Pureone Biotechnology Co., Ltd (Shanghai, China). The analytic kits for ALT and AST were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trizol was purchased from Life Technology (Carlsbad, CA). MiRNeasy mini kit and miScript PCR starter kit were obtained from Qiagen (Hilden, German). miRCURYTM LNA Array (v.18.0) was purchased from Exiqon (Vedbaek, Denmark). PrimeScript Master Mix and SYBR Premix Ex Taq were purchased from Takara (Shiga, Japan).

2.2. Experimental animals

Specific pathogen free male ICR and C57BL/6 mice (weight: 20 ± 2 g) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The mice were fed with a standard laboratory diet and given free access to tap water, living in a controlled room temperature (22 ± 1 °C), humidity (65 ± 5%) with a 12:12 h light/dark cycle. All animal experiments were performed according to the protocols approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine (Approved Number: 11002).

2.3. Treatment of animals

In the first experiment, the ICR and C57BL/6 mice were randomly divided into 3 groups, respectively. (1) Vehicle control group (n = 8), (2) EF (450 mg/kg) group (n = 8), (3) DB (300 mg/kg) (n = 8). EF and DB were dissolved in 0.5% carboxymethylcellulose sodium (CMC-Na) solution. Mice were given (intragastric administration, i.g.) with EF or DB and sacrificed at 24 h after treated with EF or DB. After treatment, blood and liver tissues from each group were collected.

In the second experiment, the ICR mice were randomly divided into 4 groups as following: (1) Vehicle control group (n = 9), (2) EF (300 mg/kg) group (n = 7), (3) EF (375 mg/kg) group (n = 9), (4) EF (450 mg/kg) group (n = 8). EF was dissolved in 0.5% CMC-Na solution. Mice were sacrificed at 24 h after EF treatment (i.g.), and blood from each group was collected.

In the third experiment, the C57BL/6 mice were randomly divided into 6 groups. (1) Vehicle control (n = 5), (2) APAP (300 mg/kg) (n = 5), (3) Vehicle control (n = 5); (4) MCT (360 mg/kg) (n = 5), (5) Vehicle control (n = 5), (6) TSN (300 mg/kg) (n = 5). APAP was dissolved in hot normal saline solution. MCT was added to distilled water and titrated to pH 3.0 with 0.1 N HCl to completely dissolve the solid. Subsequently, the solution was neutralized using 0.5 N NaOH to pH 7.0. TSN was dissolved in 10% propylene glycol solution group. Mice were given (i.g.) with APAP and sacrificed at 6 after APAP treatment. Mice were given (i.g.) with MCT and sacrificed at 48 h after MCT treatment. Mice were intraperitoneally injected with TSN and sacrificed at 24 h after TSN treatment. After treatment, blood from each group was collected.

2.4. Serum ALT/AST activity detection

The blood samples obtained from mice of all groups were allowed to coagulate for 2 h. Serum was then isolated following centrifugation at 840 × *g* for 15 min. Serum ALT and AST activities were measured with kits according to the manufacturer's instructions.

2.5. Liver histological observation

Slices of livers were fixed in 10% phosphate buffered saline (PBS)-formalin and then embedded in paraffin for histological assessment of tissue damage. Samples were subsequently sectioned (5 μm), stained with haematoxylin and eosin (H & E), and then observed under a light microscope (Olympus, Japan) to evaluate liver damage.

2.6. MicroRNA microarray and data analysis

Serum total RNA from ICR mice treated with EF or DB was harvested using Trizol and miRNeasy mini kit according to manufacturer's instructions. After having passed RNA quantity measurement using the NanoDrop 1000, the samples were labeled using the miRCURY™ Array Power Labeling kit and hybridized on the miRCURY™ Array (v.18.0). Following the washing steps the slides were scanned using the Axon GenePix 4000 B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Expressed data were normalized using the median normalization. After normalization, differentially expressed miRNAs were identified through Fold Change filtering. Only those miRNAs with the fold difference > 10.0 were considered significant, and hierarchical clustering was performed to show different miRNA expression profiling among control and EF (450 mg/kg) or DB (300 mg/kg) by using MEV software (v4.6, TIGR).

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