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Utilization of metabonomics to identify serum biomarkers in murine H_{22} hepatocarcinoma and deduce antitumor mechanism of Rhizoma Paridis saponins

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ABSTRACT

Murine H_{22} hepatocarcinoma model is so popular to be used for the preclinical anticancer candidate's evaluation. However, the metabolic biomarkers of this model were not identified. Meanwhile, Rhizoma Paridis saponins (RPS) as natural products have been found to show strong antitumor activity, while its anti-cancer mechanism is not clear. To search for potential metabolite biomarkers of this model, serum metabonomics approach was applied to detect the variation of metabolite biomarkers and the related metabolism genes and signaling pathway were used to deduce the antitumor mechanisms of RPS. As a result, ten serum metabolites were identified in twenty-four mice including healthy mice, non-treated cancer mice, RPS-treated cancer mice and RPS-treated healthy mice. RPS significantly decreased tumor weight correlates to down-regulating lactate, acetate, *N*-acetyl amino acid and glutamine signals ($p < 0.05$), which were marked metabolites screened according to the very important person (VIP), loading plot and receiver operating characteristic curve (ROC) tests. For the analysis of metabolic enzyme related genes, RPS reversed the aerobic glycolysis through activating tumor suppressor p53 and PTEN, and suppressed FASN to inhibit lipogenesis. What's more, RPS repressed Myc and GLS expression and decreased glutamine level. The regulating PI3K/Akt/mTOR and HIF-1α/Myc/Ras networks also participated in these metabolic changes. Taken together, RPS suppressed ATP product made the tumor growth slow, which indicated a good anti-cancer effect and new angle for understanding the mechanism of RPS. In conclusion, this study demonstrated that the utility of ^{1}H NMR metabolic profiles taken together with tumor weight and viscera index was a promising screening tool for evaluating the antitumor effect of candidates. In addition, RPS was a potent anticancer agent through inhibiting cancer cellular metabolism to suppress proliferation in hepatoma H_{22} tumor murine, which promoted the application of RPS in the future.

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1. Introduction

Hepatocarcinoma is a common leading cause of cancer-related death. Murine H_{22} hepatocarcinoma model is used for the preclinical anticancer candidate's screening and evaluation [1,2]. Up to now, the evaluative criteria have been mainly based on the inhibition of tumor growth. Searching for potential biomarkers from cancer animals by using a non-target metabolomics method has been proven to be a powerful tool for characterizing the pathological states in animals [3–5]. However, there were no reports using this method on the screening models such as ectopic solid tumor models and applying it for the evaluation of drugs'

Abbreviations: ALP, alkaline phosphatase; AST, aspartate transaminase; ATP5b, ATP synthase 5b; AUROC, areas under the receiver operating characteristic curves; FASN, fatty acid synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLS, glutaminase; GLUT1, glucose transporter 1; HIF, hypoxia inducible factor; HK2, hexokinase; LDHA, lactate dehydrogenase A; mTOR, mammalian target of rapamycin; MCT4, mono-carboxylate transporter proteins 4; Myc, myelocytomatosis oncogene; p53, tumor protein 53; PI3K, phosphatidylinositol 3 kinase; PKM, pyruvate kinase M; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Ras, resistance to audiogenic seizures; RPS, Rhizoma Paridis Saponins; RT-PCR, semi-quantitative reverse transcription and polymerase Chain reaction.

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antitumor effect. The NMR spectroscopic-based metabonomic technique is widely used in recent research, which can present diagnostic information and mechanistic insight into the biochemical effects of the toxins and drugs [6–9].

In this research, a metabonomics approach was applied to the murine H_{22} hepatocarcinoma model to study the metabolic features of hepatocarcinogenesis using ^{1}H NMR. The marker metabolites, which were defined from the mice model, were applied to distinguish mice bearing tumor or not, and evaluate anticancer capacity for drugs. Meanwhile, in our previous research, *Rhizoma Paridis* Saponins (RPS), which was the effective part of *Rhizoma Paridis* [10–12], showed strong anti-lung cancer and anti-hepatocarcinoma activities [13–16].

After analyzing the changes of these marker metabolites in RPS-treated mice, we tried to elucidate the pathways involved in RPS intervening tumor formation and progression.

2. Experimental procedures

2.1. Animals

Mouse hepatoma H_{22} tumor model was established in 6–8 week-old female Kunming mice. Briefly, these Kunming mice were inoculated with H_{22} tumor cells (5×10^5) s.c. in the dorsal area. All these mice were purchased from Tianjin Experimental Animal Center. All the experiments were approved by national legislations of China and local guidelines.

2.2. Drugs

The dried rhizoma of *Paris polyphylla* var. *yunnanensis* was collected in September 2010 from Lijiang, Yunnan Province, China, and identified by Professor Gao. A voucher specimen (GWCL201009) was deposited at the School of Pharmaceutical Science and Technology at Tianjin University, Tianjin, China. Rhizoma Paridis saponins (RPS) was prepared in Zhongxin Pharmaceuticals (Tianjin, China) and the method was the same as previously described [10,17]. In brief, dried crushed roots (30 kg) of *Paris polyphylla* were extracted with 70% ethanol (120 L, 3 times) for 2 h under reflux. The combined 70% ethanol extracts were concentrated and then filtered and centrifuged. The supernatant dissolved in water was then eluted by 65% ethanol on macroporous adsorptive resin D101. The eluent was finally condensed with a vacuum rotary evaporator to give a gray, viscous extract (1.5 kg), which was RPS. The agent was lyophilized and stored at −20 °C until further studies. Voucher specimens (MSCL201001) are deposited at the School of Pharmaceutical Science and Technology at Tianjin University. The steroidal saponin composition of RPS was quantitatively validated and presented elsewhere [11].

2.3. Reagents

Sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) was purchased from Merck, Germany. Acetonitrile and methanol were purchased from Sigma Aldrich, USA. Deuterium oxide (D_2O) was purchased from J&K, China.

2.4. Biochemical analyses

Serum levels of alkaline phosphatase (ALP) and aspartate transaminase (AST) were measured by the detection kits according to the manufacturer's instructions obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).

2.5. In vivo tumor growth inhibition measurement

Before carrying out the animal experiment, we measured the body weight of each mouse. Two days after injection of H_{22} cells, Kunming mice with tumors of proper size were randomly divided into two groups. The non-treated group (**Model**) was administrated orally 10 mL of 0.9% sodium chloride per 1 kg of body weight every day for 14 days. The *Rhizoma Paridis* saponins (**RPS-M**) group was administrated orally 100 mg of RPS in 10 mL of 0.9% sodium chloride per 1 kg of body weight every day for 14 days. The third group, normal group without injection of H_{22} cells received an oral administration of 10 mL of 0.9% sodium chloride per 1 kg of body weight every day for 14 days (**Normal**). In addition, the fourth group was similar to normal group but was administrated orally 100 mg of RPS in 10 mL of 0.9% sodium chloride per 1 kg of body weight every day for 14 days (**RPS-N**).

Blood samples (0.5 mL) were collected into heparinized tubes from each mouse by the puncture of the retro-orbital sinus. This was performed at the conclusion of the 14 days treatment. Blood was immediately processed for serum by centrifugation at 3500*g* for 15 min. Serum samples were frozen and maintained at −20 °C until analysis. Then all the mice were sacrificed. Autopsies were harvested. The total body weight of each mouse was firstly measured. Major organs such as liver, spleen, thymus, heart, kidney and tumor mass were then dissected and their weight was measured. The viscera index was calculated using the formula: Viscera index (mg/g) = weight of viscera (mg)/body weight (g).

2.6. Sample preparation for ^{1}H NMR spectroscopy

An aliquot of serum (250 μL) from each sample was mixed with 350 μL of an aqueous Phosphate solution (0.2 mol/L). The addition of an internal standard like sodium trimethylsilylpropionate (TSP) (100 μL) is prohibited by its interaction with proteins present in the sample. Subsequently, the total volume was transferred to a 5 mm NMR tube.

2.7. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was measured at a ^{1}H frequency of 400 MHz using an AVANCE III spectrometer, equipped with a 5-mm BBO liquid probe. Serum samples spectra were recorded using a presaturation one-dimensional preset pulse sequence with a spectral width of 6393.86 Hz digitized into 32,768 data points, resulting in an acquisition time of 2 s. A solvent presaturation suppression water spike was applied during the relaxation delay (RD) of 2 s and the mixing time of 150 ms. All spectra were calibrated (TSP peak at 0.0 ppm), then manually phased, and baseline corrected. Prior to the Fourier transformation, the free induction decays were zero-filled to 32 K and an exponential weighing factor corresponding to a line broadening of 0.5 Hz was applied. All ^{1}H NMR free induction decays (FID) were imported into MestReNova NMR Suite Version 6.0.2 software package (Mestrelab Research, S.L.) for processing and binning.

2.8. Semiquantitative reverse transcriptase polymerase chain reaction gene expression analysis

Total RNA was isolated from mice liver and tumor tissues using TRIzol (Life Technologies Inc.) according to the manufacturer's instruction. The quality of RNA was assessed by the absorbance of the samples at 260 and 280 nm cDNA synthesis was performed using Revert AidTM M-MuLV RT (Fermentas, Hanover, MD, USA) according to the supplier's protocol. Resulting reverse transcription

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