Ethanol extract of *Ardisiae Japonicae Herba* inhibits hepatoma carcinoma cell proliferation *in vitro* through regulating lipid metabolism

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1. Introduction

Hepatoma carcinoma (HCC) is the most common form of primary liver cancer, comprising 75%–85% of cases. The main risk factors for HCC are chronic infection of hepatitis B virus (HBV) and hepatitis C virus (HCV), smoking, ethanol abuse and other carcinogenic agents. Epidemiology studies have demonstrated that liver cancer ranks the fourth in terms of global mortality rate in cancer related death in 2011 (Freddie et al., 2011). Almost 500,000 people are suffering from HCC each year and half of them are from China (Elserag, 2011). Currently, surgical excision could be the best treatment for HCC. However, only<20% HCC patients received surgical excision clinically (Schwartz, Roayaie & Konstadoulakis, 2007). Transcatheter arterial chemoembolization (TACE) is commonly used as a non-surgical treatment for HCC, but TACE has many disadvantages including incomplete necrosis of tumor, multiple treatment and recurrence (Liu et al., 2015). Therefore, developing new drug therapy for HCC could increase the survival of HCC.

Accumulated evidence suggested that extract from natural herbal medicines could inhibit the proliferation of HCC. Astragaloside IV could inhibit HCC cell growth and metastasis through suppressing epithelial-mesenchymal transition (Qin et al., 2017). Polysaccharide derived from corn silk could inhibit HCC growth and increase the production of interleukine (IL)-2, IL-6 and tumor necrosis factor (TNF)-α in the HCC tumor-bearing mice (Yang et al., 2014). It also has been demonstrated that berberine could induce the cell cycle arrest in HCC cells through Akt/FoxO3a/Spk2 pathway (Li, et al., 2018). Total saponins of *Albiziae Cortex* could induce S phase arrest and activate mitochondrial apoptosis in HCC cells (Qian et al., 2018). Shikonin extracts could induce apoptosis and DNA damage in HCC cells through activating caspases and increasing intracellular Ca2+ concentration (Wang, Liu, Li, Zhao & Guan, 2017).

**Objective:** The aim of this study is to discover the possible working mechanisms of *Ardisiae Japonicae Herba* (AJH) on hepatoma carcinoma (HCC). The genomic wide RNA sequencing (RNA-seq) was performed to screen deregulated genes in HCC cells after the treatment of AJH extract. The gene and protein expression related to lipid metabolism in HCC cells were also investigated to validate the results obtained from RNA-seq.

**Methods:** In this study, ethanol extract of AJH was prepared and used to treat HCC cell in vitro. Furthermore, a genominc wide RNA sequencing (RNA-seq) was performed to screen deregulated genes in HCC cells after the treatment of AJH extract. The gene and protein expression related to lipid metabolism in HCC cells were also investigated to validate the results obtained from RNA-seq. Results: AJH extract could inhibit HCC cell proliferation *in vitro*. RNA-seq analysis has identified 1,601 differentially expressed genes (DEGs, fold change ≥ 2.0 or fold change ≤ 0.5, P < 0.05) in HCC after AJH extract treatment, which included 225 up-regulated genes and 1,376 down-regulated genes. KEGG pathway analysis of DEGs demonstrated that lipid metabolism was a potential pathway related to AJH treatment. In agreement with the RNA-seq data, qPCR and Western-blot analysis indicated that expression of genes and proteins related to lipid metabolism (SREBP1, ACC, ACLY and FASN) were significantly down-regulated in AJH treatment group as compared with the control group. Furthermore, AJH extract could also decrease lipid contents and cellular free fatty acid levels in HCC cells.

**Conclusion:** Ethanol extract of AJH could inhibit HCC cell proliferation *in vitro*, the possible mechanism may be related to the inhibition of lipid metabolism.

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Ardisiae Japonicae Herba (AJH), native to South East Asia and North America, has been widely used to treat HCC, bronchitis conjunctivitis and trauma for thousands of years (Kobayashi & de Mejia, 2005). Clinical studies demonstrated that Chinese herbal formula containing AJH, Scutellaria barbata D. Don, Hedyotis diffusa Wild, and other 23 herbs could improve the clinical outcome and induce the quality of life in HCC patients (Zeng et al., 2019). High-performance liquid chromatography (HPLC) analysis indicated that the chemical constituents in AJH were coumarins, flavonoid glycosides, catechins, bergenin and quercitrin, with the later two components identified as the most abundant compound monomers in the AJH (Yu et al., 2017). However, few researches were carried out to study the effects and mechanisms of the constituents in AJH on HCC. In this study, ethanol extract of AJH was prepared and used to treat HCC cell in vitro. In addition, RNA sequencing (RNA-seq) was used to screen the deregulated genes in the HCC cell after AJH extract treatment. The lipid metabolism in HCC cells was also investigated to validate the results obtained from RNA-seq.

2. Materials and methods

2.1. Reagents

HCC cell line (Hep3B) was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China); Modified Eagle’s Medium (MEM) and penicillin/streptomycin (PS) were purchased from Gibco Biotechnology Co., Ltd. (Beijing, China); Fetal bovine serum (FBS) was purchased from Hyclone Bioscience Co., Ltd. (Beijing, China). Extract total RNA, first-stand cDNA reverse transcription and polymerase chain reaction kits were purchased from TianGen Biotechnology Co., Ltd. (Beijing, China); Cellular free fatty acid test kit was purchased from Solarbio Biotechnology Co., Ltd. (Beijing, China). Rabbit anti-ACC (ab45174) and rabbit anti-ACLY (ab40793) and rabbit anti-SREBP1 (bs-1402R) was purchased from Bioss Technology Co., Ltd. (Beijing, China); BCA test kit was purchased from Solarbio Biotechnology Co., Ltd. (Beijing, China); BCA test kit was purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Beijing, China).

2.2. Preparation of ethanol extract of AJH

The ethanol extract of AJH was prepared based on the ultrasonic ethanol method as described previously (Wang et al., 2018). Briefly, 5 g of AJH were weighed and blended with 75 mL of 90% ethanol (solild–liquid ratio = 1:1.5 g/mL). Then, the mixture was exposed to 120 W ultrasonic wave for 90 min. The supernatant liquid was collected followed by filtration and evaporation to obtain the ethanol extract of AJH.

2.3. Cell culture

Hep3B cell line was cultured in T75 tissue culture flasks with 15 mL of medium (prepared with 89% MEM, 10% FBS and 1% PS). The tissue culture flasks were placed in an incubator with saturated humidity at 37 °C containing 5% CO2. Fresh medium was replaced every 24 h.

2.4. Cell proliferation assay

Cell proliferation assay after AJH extract treatment was performed using MTT method as described previously (Ferreira et al., 2008). Briefly, Hep3B cells were seeded in a 96-well plate at the concentration of (3 × 10^4) cells/well. Cells were then incubated with different concentrations (0, 5, 10, 20, 50 µg/mL) of AJH extract for 24 h and 48 h, respectively. Cells were subsequently incubated with MTT [3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyl tetrazoliumbromide] for 3 h. After incubation, the media in each well were removed and the cells were resorbed by DMSO. The OD value was measured at 490 nm using a microplate reader (Varioskan Flash, Thermo).

2.5. RNA-seq

Total RNAs were extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer’s protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples with RNA Integrity Number (RIN) > 7 were subjected to the subsequent analysis. The libraries were constructed using TrueSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. Then these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500 or IlluminaHiSeq X Ten) and 125 bp/150 bp paired-end reads were generated.

2.6. Bioinformatic analysis of RNA-seq

Raw data (raw reads) were processed using Trimomatic (Bolger, Lohse & Usadel, 2014). The reads containing poly-N and the low quality reads were removed to obtain the clean reads. Then the clean reads were mapped to reference genome using hisat2 (Kim, Langmead & Salzberg, 2015). FPKM (Kim, Langmead & Salzberg, 2015) and read counts value of each transcript (Protein-coding) was calculated using bowtie 2 (Langmead & Salzberg, 2012) and eXpress (Roberts & Pachter, 2012). Differentially expressed genes (DEGs) were identified using the DESeq (Love, Huber & Anders, 2014) functions estimate Size Factors and nbinom Test. P-value < 0.05 and fold change (FC) > 2 or FC < 0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of DEGs was performed to explore transcripts expression pattern. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of DEGs was performed using R based on the hypergeometric distribution (Minoura et al., 2008).

2.7. Real-time reverse transcription quantitative polymerase chain reaction (qPCR)

qPCR was conducted as described previously (Ami et al., 2012). In brief, total RNA was isolated using the extract RNA kit and first strand cDNA was synthesized from 1 µg of total RNA according to the manufacturer’s instructions. qPCR was used to detect the expression of SREBP1, ACC, ACLY and FASN. All samples were run in triplicate and detected by BIORad iQ5. HPRT was used as a loading control. Relative quantification was done using the 2^-ΔΔCT method (Livak & Schmittgen, 2000). The sequences of primers used were listed in Table 1.

2.8. Western-blot

Protein samples were isolated and normalized using a BCA protein assay kit. For western blotting, protein samples were loaded onto 5% (ACC), 7.5% (ACLY) and 15% (SREPP1, FASN and β-actin) SDS-PAGE, then transferred onto a nitrocellulose membrane and blocked with 5% BSA in Tween-Tris-buffered saline (TBST) solution. The membrane was incubated with primary antibody (rabbit anti-SREBP1 1:2000, rabbit anti-ACC 1:5000, rabbit anti-ACLY 1:5000, rabbit anti-FASN 1:5000, rabbit anti-β-actin 1:1000) in 1% BSA/
TBST at 4 °C overnight, followed by secondary antibody probing (HRP-conjugate goat anti-rabbit IgG 1:4000). The blots were visualized using chemiluminescence (Fusion FX, VILBERLOURMAT, France) following the manufacturer's protocol. The intensity of the bands was determined using the Image J software.

### 2.9. Oil Red O staining

Cells were washed with PBS and fixed in 4% paraformaldehyde for 40 min. After removing the fixative, cells were washed with PBS and then stained with Oil Red O following the manufacturer's instructions (Solarbio). The staining extent of Oil Red O was detected and quantified using Image Pro Plus 6.0. The integrated optical density (IOD) was then observed. The mean optical density (MOD) was calculated according to the following formula: MOD = IOD/sum area.

### 2.10. Investigation of cellular free fatty acid

Briefly, 1 × 10^6 cells were diluted into 300 μL of normal saline followed by ultrasonic trituration and centrifugation at 3000 rpm for 15 min to obtain cellular homogenate. The levels of cellular free fatty acid were investigated according to the manufacturer's instructions. Cellular homogenates were normalized to total protein content as detected by BCA assay (Nanjing Jiancheng Bioengineering Institute).

### 2.11. Statistics

Data were analyzed with a mean ± standard deviation (mean ± SD) for the independent experiments. Statistical differences between the experimental groups were examined by analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was determined at a F-value < 0.05, using SPSS version 20.0. Curve-fitting was carried out using the graphical package GraphPad Prism5.

# 3. Results

### 3.1. Cell viability in HCC cells treated with AJH extract

To investigate whether AJH extract could inhibit HCC cell proliferation, HCC cells were treated with different concentrations of AJH extract in vitro and cell viability were measured using MTT assay. After 24 and 48 h of treatment, the cell viability of HCC was decreased in a concentration dependent manner. The inhibition rates of HCC after AJH extract treatment at different concentrations were shown in Fig. 1. The results demonstrated that AJH extract could significantly inhibit HCC proliferation at the minimum concentration (5 μg/mL, incubated for 48 h), which was then selected for the following studies.

### 3.2. Identification of DEGs and relative pathways in HCC cells after treated with AJH extract

HCC cells were seeded in a 6-well plate at the concentration of 5 × 10^5 cells/well and incubated with or without AJH extract (5 μg/mL) for 48 h. DEGs in HCC cells were screened using RNA-seq. Generally, 1,601 DEGs (FC ≥ 2.0 or FC ≤ 0.5, P < 0.05) were identified in HCC cells after AJH extract treatment, including 225 up-regulated genes and 1,376 down-regulated genes (Fig. 2A). Moreover, clustering analysis showed that the classification of DEGs in sequencing data were in agreement with the experimental groups (Fig. 2B).

Pathways of AJH on HCC cell proliferation were predicted using KEGG analysis. The predicted pathway terms could be classified into cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems (Fig. 2C). HCC cells have been demonstrated with the dysfunction of lipid metabolism. Regulating the lipid metabolism could inhibit the progression of HCC (Luo, Hong, Lu, Qiu & Fan, 2016; Osugi et al., 2015). Furthermore, studies have demonstrated that main components in AJH such as bergenin (Jahromi et al., 2010), quercitrin (Choi et al., 2010) and catechins (Murase et al., 2002) could regulate lipid metabolism. Likewise, our unpublished data indicated that AJH could reduce the body weight in obese mice. Thus, lipid metabolism was predicted to be a potential pathway related to AJH treatment.

### 3.3. Effects of AJH extract on lipid metabolism in HCC cells

According to the deep sequencing data, AJH extract may affect lipid metabolism in HCC cells. We further validated the deep sequencing data using qPCR, Western-blot, Oil Red O staining and cellular free fatty acid content measurement. Among the 32 DEGs (28 down-regulated genes and four up-regulated) related to lipid metabolism, SREBP1 (You et al., 2018), ACC (Depertalita et al., 2014), ACY (Wu et al., 2015) and FASN (Wu et al., 2015), which could be involved in HCC progression, were selected for the validation of RNA-seq results. In agreement with the RNA-seq data, gene expressions of SREBP1, ACC, ACY and FASN were significantly down-regulated in AJH group as compared with the control group (P < 0.01 respectively Fig. 3A). Moreover, Western-blot analysis also indicated that AJH treatment inhibited the maturation of SREBP-1 and decreased the protein levels of ACC, ACY and FASN, which was in agreement with the sequencing data (P < 0.01 respectively Fig. 3B – F). Also, Oil Red O staining showed a significant

### Table 1

| Genes | Primer sequences (5'→3') |
|-------|--------------------------|
| HPRT  | Forward: TGACACTGGAAAAACATGGCA  
Reverse: CGCTTTCCTGACGAGGACCT |
| SREBP1| Forward: CAGCAGGTTTGCAGGAAAGT  
Reverse: CGCTTCAGTGGCCTGTTG |
| ACC   | Forward: AACCGGAAAAGTGAGGAGG  
Reverse: TCTCGGAGGCAGCATACA |
| ACY   | Forward: AGCGCATGGCCTGATGAG  
Reverse: GCAAGATGGCTGCTGAGT |
| FASN  | Forward: CGCGTGGAGGCCGTCCTAC  
Reverse: CGGGCTGCAACCAGGTCTCT |

GraphPad Prism5.

**Fig. 1.** AJH extract inhibited HCC cell proliferation in vitro (mean ± SD, n = 6). HCC cells were treated with AJH extract at different concentrations (0, 5, 10, 20, 50 μg/mL) for 24 h and 48 h respectively. Cell proliferation was investigated using MTT assay. *P < 0.01 compared with 0 μg/mL.
decrease of lipid contents in AJH group compared with the control group \((P < 0.01, \text{Fig. 4A and 4B})\). The level of cellular free fatty acid was also decreased in AJH group as compared with control group \((P < 0.01, \text{Fig. 4C})\).

4. Discussion

In this study, it was demonstrated that ethanol extract of AJH could inhibit HCC cell proliferation, indicated an anti-tumor poten-
AJH extract. The possible mechanisms of AJH extract inhibiting HCC cell proliferation were tested using RNA-seq, which is an effective approach that offers massive information to identify DEGs (Marioni et al., 2008). This approach also contains comprehensive gene expression data that can be used to discover the molecular mechanism of drugs by analyzing gene expression changes. Our current study showed that more than 1,600 genes were deregulated in HCC cells after AJH treatment, indicating that AJH could significantly affect HCC cells in transcriptional levels. In addition, KEGG analysis of DEGs indicated that lipid metabolism pathway may be related to the antitumor effects of AJH.

Cellular lipids, which are major components of organelles and cell membrane, include phospholipid, fatty acid, triglyceride, cholesterol and cholesteryl ester (Holthuis & Menon, 2014; Mukherjee & Maxfield, 2004; Van, 2010). Lipids could also serve as second messengers participating in cell signaling and energy resources in response to nutrient scarcity (Efeyan, Comb & Sabatini, 2015; Guo, Bell & Chakravarti, 2013). Glucose is the major resource for lipid synthesis. In the pay off phase of glycolysis, the carbon skeleton of glucose is degraded to pyruvate which is oxidized to acetyl group and is in turn formed into citrate in mitochondria. The citric acid is released into cytoplasm to generate acetyl-CoA, which is catalyzed by ATP citrate lyase (ACLY). Acetyl-CoA is then carboxylated to form malonyl-CoA under the catalysis of acetyl-CoA carboxylase (ACC). Finally, fatty acid synthase (FASN) catalyzes the transformation of acetyl-CoA into fatty acid. Many studies have demonstrated the dysfunction of lipid metabolism in tumor cells (Pascual, Augustinova, Mejetta, Mercè Martin & Benitah, 2017; Zhao et al., 2017). Tumor cells require many lipids to synthesis cell membrane components (Röhrig & Schulze, 2016). The expressions and activities of ACLY, ACC and FASN were significant higher in tumor cells (Khwairakpam et al., 2015; Menendez et al., 2004; Osugi et al., 2015; Su et al., 2014). Decreasing the activities of these enzymes could inhibit tumor growth (Luo, Hong, Lu, Qiu & Fan, 2016; Mullen & Yet, 2015). The current study also demonstrated that AJH extract could decrease both gene and protein levels of ACLY, ACC, FASN and the levels of cellular free fatty acid composition, which indicated that AJH extract could inhibit the lipid metabolism in HCC.

In addition, SREBP1 was also down-regulated and maturation of SREBP1 was decreased in HCC cells after AJH treatment. SREBP1 is a subset of sterol regulatory element-binding proteins (SREBPs), which is a key transcriptional factor involving in the biosynthesis and uptake of fatty acid and cholesterol (Goldstein, Debose-Boyd & Brown, 2006; Goldstein & Brown, 2015; Nohturfft & Zhang, 2009). SREBPs, together with SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein (Insig), could form a complex in endoplasmic reticulum (ER) (Sun, Li, Goldstein, & Brown, 2005). The precursor SREBP1 (pSREBP1) could be activated after the N-glycosylation of SCAP following transported to golgi and transformed into mature SREBF-2 (mSREBP1) (Cheng, Ru, Geng, Liu & Guo, 2015; Sun, Seemann, Goldstein & Brown, 2007). The mSREBP1 could enter cell nucleus and regulate the transcription of lipid metabolic enzyme (Moon et al., 2000; Miyamoto et al., 2000).

5. Conclusion

In conclusion, this current study demonstrated that ethanol extract of AJH could inhibit HCC cell proliferation in vitro, the possible mechanism may be related to the inhibition of lipid metabolism. This study could open avenues of using AJH to treat HCC. The antitumor effects of AJH will be verified in other HCC cell lines and using animal models in our future studies. Decreased lipid contents was observed in HCC cells after AJH treatment, metabolomics and lipidomics should be carried out to deeply illustrate the modulatory effects of AJH on lipid metabolism. In addition, results in the current study showed that AJH treatment inhibited the activation of SREBP signaling pathway in HCC cells, gene stimulation, silencing and molecular docking methods will be applied in further studies to elucidate the detailed antitumor mechanisms of AJH on HCC. Furthermore, phosphatidylglycerophosphate synthase 1 (PGS1), lanosterol synthase (LSS), lipin1 (LPPN1) and glucosylceramidase beta 2 (GBA2), which were associated with lipid metabolism, were up-regulated after AJH treatment. Few studies have demonstrated the role of PGS1, LSS, LPPN1 and GBA2 in the progression of HCC. It still needs further studies to elucidate modulatory effects of these genes on HCC proliferation and SREBP signaling pathway.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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