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Research paper

# The altered microRNA profile in andrographolide-induced inhibition of hepatoma tumor growth

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A B S T R A C T

*Background:* MicroRNAs (miRNAs) have been reported to play critical roles in regulating gene expression in tumor development. Natural compound andrographolide (Andro), isolated from medicinal herb *Andrographis paniculata*, was reported to inhibit hepatoma tumor growth in our previous studies. The present study aims to observe the altered miRNAs profile and related signaling pathways involved in Andro-induced inhibition on hepatoma tumor growth.
*Results:* The inhibition on hepatoma tumor growth induced by Andro (10 mg/kg) was found in a xenograft mouse tumor model *in vivo*. The results of miRNAs chip analysis showed that the expression of 22 miRNAs was increased, whereas the expression of other 10 miRNAs was decreased after Andro treatment. Further, the increased expression of miR-222-3p, miR-106b-5p, miR-30b-5p, and miR-23a-5p was confirmed in hepatoma Hep3B and SMCC7721 cells *in vitro* after cells were treated with Andro (50 μM) for the indicated time. Functional annotation of the target genes based on the differentially expressed miRNAs demonstrated that the majority of the genes were involved in a variety of signaling pathways, including miRNAs in cancer, mitogen-activated protein kinases (MPAKs), focal adhesion. Furthermore, the expression of 24 target genes (total 31) involved in above signaling pathways based on miRNAs analysis was found to be consistent with the alteration of miRNAs.
*Conclusions:* The results demonstrate that Andro alters the expression of miRNAs profile and downstream signals, which may contribute to its inhibition on hepatoma tumor growth.

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## 1. Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNA molecule, containing about 22 nucleotides. A large number of studies show that miRNAs have crucial roles in transcriptional and post-transcriptional regulation of cellular gene expression (Chen and Rajewsky, 2007). Increasing evidences demonstrate the critical roles of miRNAs in many human physiological and pathological processes, such as cancer development, cellular immune response, etc. (Huang et al., 2014; Lee et al., 2014; Peng et al., 2014). Hepatocellular carcinoma (HCC) is one of the most common and deadly malignancies in the world. Up to now, the strategy for HCC treatment and the prognosis of HCC in patients remain poor. Increasing reports demonstrate that miRNAs play important roles in carcinogenesis and progression of HCC, and can be the biomarker for the prognosis of HCC (Borel et al., 2012; Giordano and columbano, 2013; Wong et al., 2013; Hung et al., 2014). In addition, some reports demonstrate that miRNAs are the promising therapeutic target for HCC (Drakaki et al., 2013; Ling et al., 2013; Li et al., 2014).

Andrographolide (Andro) is a main natural compound in medicinal herb *Andrographis paniculata*, which is traditionally used for anti-inflammatory, anti-infection in clinic in China. Previous studies in our lab and some other groups have already evidenced the inhibition of Andro on HCC growth *in vivo* and *in vitro* (Ji et al., 2007, 2009, 2011; Yang et al., 2009; Chen et al., 2012; Shen et al., 2014). Increasing evidences demonstrate that miRNAs play important roles in regulating the anti-cancer function of some natural compounds such as curcumin, genistein, 3,6-dihydroxyflavone, diaporine A (Saini et al., 2011; Chang et al., 2012; Xia et al., 2012; Song et al., 2014). However, there is still no related report about the effects of Andro on miRNA expression profile when it exerts its inhibition on hepatoma tumor growth.

In this present study, we observed the altered miRNAs profile in hepatoma tumor tissues in Andro-treated nude mice *in vivo*, and found some changed miRNAs, which was further confirmed in hepatoma Hep3B and SMCC7721 cells *in vitro*. In addition, the expression of target

*Abbreviations:* Andro, andrographolide; ERK, extracellular regulated kinase; HCC, Hepatocellular carcinoma; HPLC, high pressure liquid chromatography; JNK, c-Jun N-terminal kinase; KEGG, Kyoto Encyclopedia Genes $amp Genomes; MPAKs, mitogen-activated protein kinases; miRNAs, microRNAs; Tregs, regulatory T cells; NSCLC, non-small-cell lung cancer; SEM, standard error of the mean.

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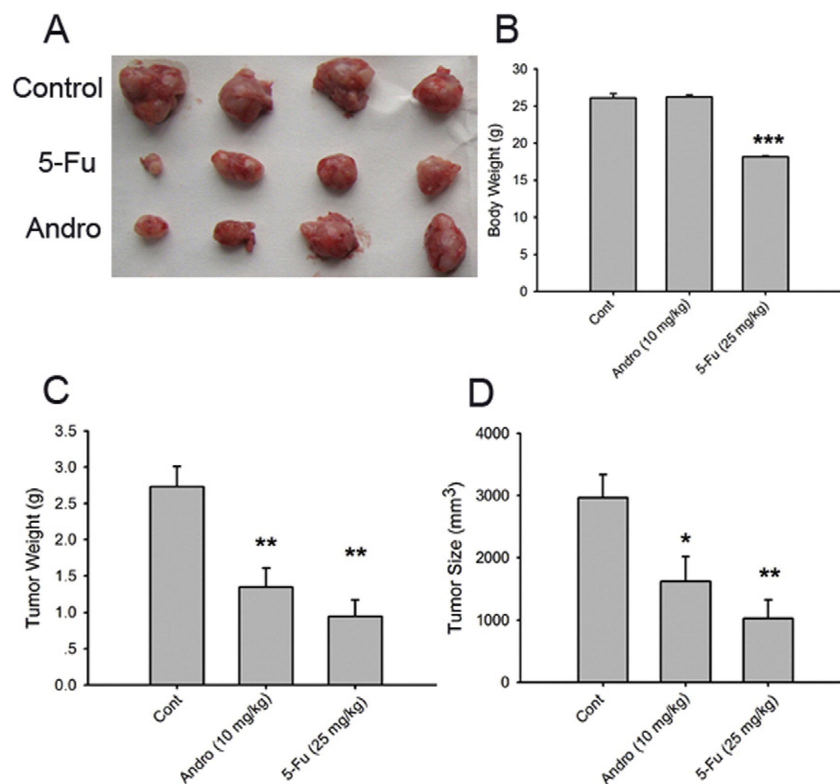


**Fig. 1.** Andrographolide (Andro) inhibited the tumor growth in nude mice bearing hepatoma Hep3B cells. (A) Pictures of tumor. (B) Body weight of mice. (C) Tumor weight. (D) Tumor size. Data are shown as means ± SEM (n = 4), $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with control.

genes involved in the signaling pathways based on miRNAs analysis was further detected.

## 2. Materials and methods

### 2.1. Chemical compounds and reagents

Andrographolide (Andro) was purchased from Nanjing TCM Institute of Chinese Materia Medica (Nanjing, China). The purity of the compound was over 98.5% as determined by high pressure liquid chromatography (HPLC) analysis.

Trizol reagent was purchased from Life Technology (Carlsbad, *CA*). miRNeasy mini kit was obtained from Qiagen (Hilden, German). miRCURYTM LNA Array (v.18.0) was purchased from Exiqon (Vedbaek, Denmark). Reverse transcriptase and qPCR mix kits for miRNA analysis were obtained from TOYOBOS (Osaka, Japan). PrimeScript$^{RT}$ Master Mix and SYBR Premix Ex Taq™ were purchased from Takara (Shiga, Japan).

### 2.2. Cell lines and culture

Hepatoma-derived Hep3B cell line was obtained from the American Type Culture Collection (Manassas, VA). Hepatoma-derived SMCC7721 cell line was purchased from Cell Bank, Type Culture Collection of Chinese Academy of Sciences (Shanghai). Cells were cultured in MEM media supplemented with 10% [v/v] heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

### 2.3. In Vivo tumor xenograft assay

Specific pathogen-free nude male mice (6–8 wk. of age) were obtained from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All animal experiments were performed according to the protocol approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

Mice were subcutaneously injected of Hep3B cells ($1 \times 10^6$ cells per mouse) into the left front leg. Andro (10 mg/kg) was intra-peritoneally injected into the mice daily and solvent control was also injected into normal mice. After administration of Andro for 16 days, the mice were sacrificed and tumors were removed, weighed and photographed. The tumor sizes were determined by Vernier caliper measurements and calculated as $[(\text{length} \times \text{width}^2)/2]$.

### 2.4. MicroRNA microarray and data analysis

Total RNA 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™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.18.0). Following the washing steps the slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs that intensities ≥30 in all samples were chosen for calculating normalization factor. 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 >2.0 and $P$ value <0.05 were considered significant. Finally, hierarchical clustering was performed to show distinguishable miRNA expression profiling among samples by using MEV software (v4.6, TIGR).

### 2.5. miRNA qRT-PCR.

Hep3B or SMCC7721 cells were incubated with Andro (50 μM) for 12 h and 24 h. Cellular total RNA was isolated by Trizol plus miRNeasy mini kit according to manufacturer's instructions. The RNA content

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