RESEARCH ARTICLE

Anti-proliferative Effects of *Atractylis lancea (Thunb.) DC.* via Down-regulation of the c-myc/hTERT/Telomerase Pathway in Hep-G2 Cells

Wei-Qiang Guo*, Liang-Zhi Li, Zhuo-Yang He, Qi Zhang, Jia Liu, Cui-Ying Hu, Fen-Ju Qin, Tao-Yun Wang

Abstract

***Atractylis lancea* (*Thunb.*) *DC.* (AL), an important medicinal herb in Asia, has been shown to have anti-tumor effects on cancer cells, but the involved mechanisms are poorly understood. This study focused on potential effects and molecular mechanisms of AL on the proliferation of the Hep-G2 liver cancer cell line *in vitro*. Cell viability was assessed by MTT test in Hep-G2 cells incubated with an ethanol extract of AL. Then, the effects of AL on apoptosis and cell cycle progression were determined by flow cytometry. Telomeric repeat amplification protocol (TRAP) assays was performed to investigate telomerase activity. The mRNA and protein expression of human telomerase reverse transcriptase (hTERT) and c-myc were determined by real-time RT-PCR and Western blotting. Our results show that AL effectively inhibits proliferation in Hep-G2 cells in a concentration- and time-dependent manner. When Hep-G2 cells were treated with AL after 48h,the IC_{50} was about 72.1 μg/mL. Apoptosis was induced by AL via arresting the cells in the G1 phase. Furthermore, AL effectively reduced telomerase activity through inhibition of mRNA and protein expression of hTERT and c-myc. Hence, these data demonstrate that AL exerts anti-proliferative effects in Hep-G2 cells via down-regulation of the c-myc/hTERT/telomerase pathway.**

Keywords: *Atractylis lancea (Thunb.) DC* - liver cancer - telomerase activity - hTERT - C-myc

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Introduction

Liver cancer, a major health problem, is the 6th most common cancer in the world. Chinese liver cancer patients accounts for approximately more than 50% globe (Feo et al., 2008; Fabregat, 2009). Except of surgery, radiotherapy and chemotherapy, Chinese medicinal herbs and traditional Chinese medicines have been applied in liver cancer therapy because of their low toxicity and effective therapeutic benefit (Mok et al., 2007).

Telomeres, DNA-protein structures at the ends of chromosomes, are essential for chromosome protection and genome stability in human (Moyzis et al., 1988). In human somatic cells, telomeres shorten which are attributed to down-regulation of telomerase, causes chromosome fusions lossing, cell cycle arrest and apoptosis. However, telomerase are active in more than 85% of cancer tumors (Murnane, 2010). These findings imply that activation of telomerase is associated with the development of cancer tumors (Donate & Blasco, 2011). Human telomerase reverse transcriptase (hTERT), one catalytic subunit of Human telomerase, is the pivotal factor in regulation of telomerase activity (Shay & Wright, 2011). The up-regulation of hTERT is the key step for enhancing telomerase activity, which is required for immortalization and development of cancer. Thus, hTERT may be an excellent candidate for cancer therapy (Ouellette et al., 2011; Sprouse et al., 2012).

Atractylis lancea (Thunb.) DC. (AL), an important medicinal herb in China, has a long history in therapy of several diseases, such as removing wind and darnpness, promoting eyesight (Liu et al., 2012; Peng et al., 2012). Recent studies suggest that AL possesses anti-cancer property in a wide range of cancer such as hepatocarcinoma and gastric in vivo and vitro (Mahavorasirikul et al., 2010; Zhao et al., 2013). However, the possible anti-cancer mechanisms of AL are not well understood. Therefore, we investigated the effects of AL on telomerase activity and associated pathway in human liver cancer cell line Hep-G2.

Materials and Methods

Cell culture

Human liver cancer Hep-G2 cells were maintained in DMEM high sugar medium supplement with 10% fetal

*School of Chemistry, Biology and Material Engineering, Suzhou University of Science and Technology, Suzhou, Jiang Su Province, China *For correspondence: weiqiang.guo@hotmail.com*

Table 1. The Sequences of PRIMERS USed in Real-time RT-PCR Analysis

Gene	Sequence
hTERT	F: 5'-ACCCTCTACAAGTGCTATCTGA-3'
	R: 5'-CAAGTTCCTGGACTGGCTGAA-3'
c-myc	F: 5'-GCTCCGTTTTACCTCGTGCC-3'
	R: 5'-TGCTGCCAAGCGGGTGAAGT-3'
GAPDH	F: 5'-ATGAGTCCTTCCACGATACCAA-3'
	R: 5'-AATGCCTCCTGCTCCACCAA-3'

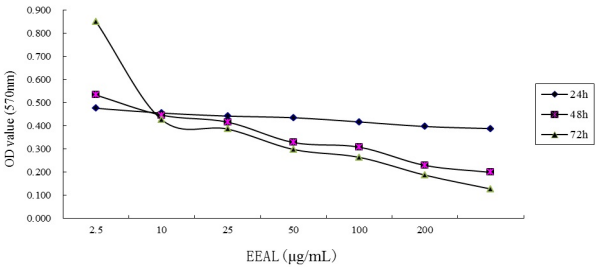

Figure 1. Effect of AL on the Cell Viability by MTT Assay. Hep-G2 cells were treated with AL at final (from 2.5 μg/mL to 250 μg/mL) for 24, 48 and 72 h, respectively

calf serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, USA) at 37 °C in 5% CO_2 and 100% humidity.

*Ethanol extract of **Atractylis lancea (Thunb.) DC.** (EEAL)*

AL was oven-dried at 55°C until stability of dry weight, then ground into powder. Extraction of AL was carried out by ultrasounding the powder (50 g) containing 500 mL of 95% ethanol. The extracted solvent was centrifuged for 20 min in 5000 rpm, and then collected the supernatant. The supernatant was evaporated by freezer dryer. The extracts were dissolved in DMEM medium and stored at-20°C. The components of extract were analyzed by HPLC.

Cell viability assay

Hep-G2 cells were seeded in 96-well plates (10^4/well). After 6 h, different concentrations of EEAL (from 1 μg/mL to 250 μg/mL) were added. When the cells were treated with EEAL for 24 h, 48 h, 72 h, respectively, 20 μL of MTT solution (5 mg/mL) was added to each well for 4 h at 37 °C. Then, 100 μL of DMSO was added into each well for another 10 min. The optical density (OD) was measured by microplate reader at 570 nm. Each assay was performed triplicate.

Apoptosis assay by flow cytometry

Hep-G2 cells were seeded in 6-well plates (10^6/well). When cells were in logarithmic growth period, three concentrations of EEAL (obtained from the result of cell viability: 5 μg/mL, 50 μg/mL, 200 μg/mL) were added. After treated for 48 h, cells were harvested with trypsinase and washed twice with PBS. According to manufacturer's instruction, cells were stained with TUNEL kit (Roche, Germany) then processed by flow cytometry. Data acquisition and analysis were done on BD FACSCaliber using cellquest software.

Assay of telomerase activity

Hep-G2 cells, treatment with EEAL (5 μg/mL, 50 μg/mL, 200 μg/mL) for 48 h, were trysinized and washed with cold PBS, and then incubated in lysis buffer for 30 min at -80 °C. The lysates were centrifuged at 15,000 g for 40 min and protein concentrations of the supernatant were measured using Bradford assay. Telomerase activity was determined by TRAP-PCR (Ohyashiki et al., 1997), the TRAP-PCR reaction mixture contained 0.5 μg protein of each sample, 10 pM of each primer (Ts: 5'-AATCCGTCGAGCAGAGTTAGGGTTAG-3'; Cx: 5'-CCCTTACCCTTACCCTTACCCTAA-3'), 10 mM dNTPs, 1 U Taq DNA polymerase (Roche, Germany), 1× Taq PCR buffer. The TRAP-PCR amplification conditions included denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min. This cycle was repeated for 30 times. The PCR products were separated by PAGE gel electrophoresis and stained with silver to analyze the degree of telomeric repeats.

Real-time reverse transcriptional polymerase chain reaction (real-time RT-PCR)

Total RNA from Hep-G2 cells incubated with EEAL (5 μg/mL, 50 μg/mL, 200 μg/mL) for 48 h, was isolated using RNA extract kit (Roche, Germany) according to the manufacturer instruction. First stand cDNA was synthetized using M-MLV reverse transcriptase (Promega, USA) in accordance with the protocol. Real-time PCR was performed using 5 μL cDNA as template, 10pM of each primer (shown in Table 1), 1× SYBR Green Taq Mixture (Roche, Germany). The real-time PCR conditions were as follows: 40 cycles at 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative mRNA expression using GAPDH as the endogenous control.

Western Blot analysis

Hep-G2 cells, incubated with EEAL (5 μg/mL, 50 μg/mL, 200 μg/mL) for 48 h, were washed with cold PBS, then were incubated with protein extract buffer for 30 min, at -80 °C. The lysates were centrifuged at 15,000 g for 40 min and protein concentration was measured by Bradford assay. Protein lysates (50 μg) from each sample were subjected to SDS-PAGE on 15% acrylamide gel and transferred to the PVDF membrane. Blots were incubated with 5% nonfat milk to block nonspecific binding sites at 4 °C overnight and then incubated with antibodies against hTERT, c-myc and GAPDH (Santa Cruz, USA) for 1.5 h at 37 °C. Membranes were then incubated in HRP-conjugated secondary antibodies (Santa Cruz, USA) for 1 h at 37 °C. Finally, membranes were detected by using ECL kit (Thermo Fisher, USA).

Statistical analysis

The data were analyzed by SPSS15.0 and the results were expressed as mean±SD. Statistical analysis of the results was performed through t-test for pairwise comparison. $P<0.05$ was considered significant.

Results

AL inhibits cell proliferation in Hep-G2 cells

AL significantly reduces growth of Hep-G2 cells in

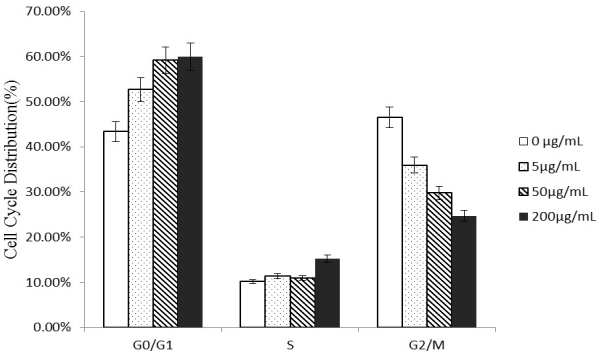

Figure 2. The Effect of AL on Cell Cycle Progression in Hep-G2 Cells Incubated with EEAL at 0, 5, 50 and 200 μg/mL for 48 h

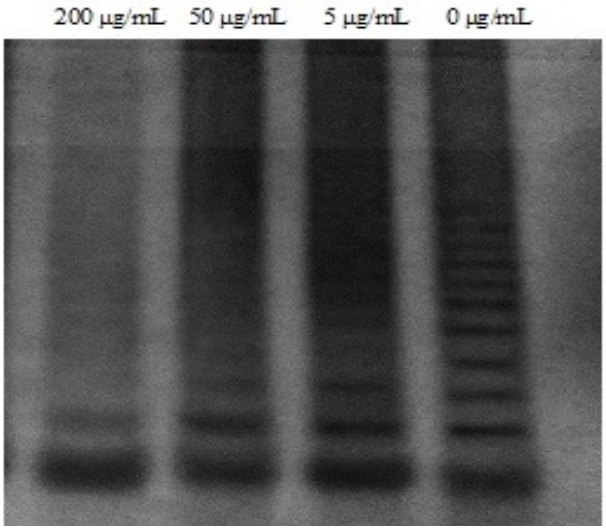

Figure 3. The Effect of AL on Telomerase Activity. TRAP-PCR assays were carried out to detect the telomerase in Hep-G2 cells. Hep-G2 cells were treated with EEAL at 0, 5, 50 and 200 μg/mL for 48 h, respectively

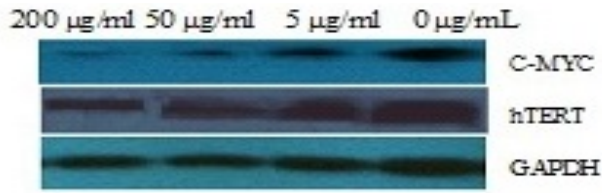

Figure 5. The Effect of AL on the Protein Expression of hTERT and c-myc by Western Blot. Hep-G2 cells were treated with EEAL at 0, 5, 50, 200 μg/mL for 48 h, respectively. GAPDH was used as internal control

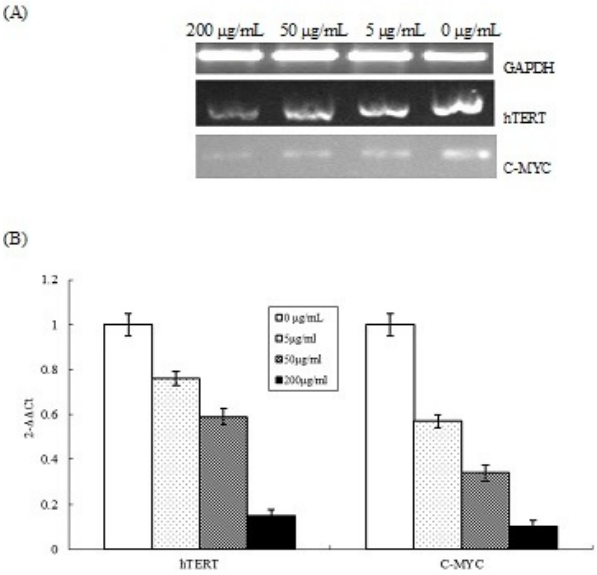

Figure 4. The Effect of AL on the mRNA Expression of hTERT and c-myc by Real-time RT-PCR. Hep-G2 cells were treated with EEAL at 0, 5, 50, 200 μg/mL for 48 h, respectively. A. Gel shows the effect of AL on hTERT and c-myc mRNA in Hep-G2 cells, measured after 48 h of treatment. GAPDH was used as internal control. B. The graph shows the difference in term of gene expression analyzing by $2^{-\Delta\Delta Ct}$

a time- and dose- dependent manner (P<0.05) (Figure 1). When Hep-G2 cells were incubated with 50 μg/mL and 100 μg/mL EEAL for 24 h, the inhibitory ratios were 11.03% and 22.46%, respectively; when the same concentrations treatment for 48 h, the inhibitory ratios were 43.58% and 59.63%, respectively. The IC_{50} value is about 72.12 μg/mL in Hep-G2 cells incubated with EEAL for 48 h.

AL induces apoptosis and arrests cell cycle at G1 phase in Hep-G2 cells

We investigated whether AL affected cell cycle progression and apoptosis in Hep-G2 cells, the results showed an effective decrease in the number of cells in S and G2/M phases and increase in the number of cells in G1 phases (Figure 2). Furthermore, we saw a marked concentration-dependent increase in G1 phase. These findings indicate that cell cycle arrest at G1 phase in Hep-G2 cells treatment with AL. To confirm these results, the effect of AL on apoptosis was evaluated. We observed a marked increase in both early and late stage apoptosis, as examined by flow cytometry, in Hep-G2 cells after AL treatment compared with normal Hep-G2 cells (Data no shown).

AL reduces the telomerase activity in Hep-G2 cells

The effect of AL on telomerase activity was determined by TRAP-PCR in Hep-G2 cells incubated with AL for 48 h. Based on the results obtained in terms of cell viability, we determined to use three concentrations of 5 μg/mL, 50 μg/mL, 200 μg/mL of EEAL. As shown in Figure 3, the 6 bp (TTAGGG) repeat length were reduced by different concentrations of AL, the inhibitory effect of 200 μg/mL AL on telomerase activity was better than 5 μg/mL and 50 μg/mL. These results suggest that AL marked inhibited telomerase activity in a dose-dependent manner.

AL decreases the mRNA and protein expression of c-myc and hTERT in Hep-G2 cells

To determine whether c-myc and hTERT are involved in the mechanism of the inhibition of telomerase activity by AL, the mRNA and protein expressions of c-myc and hTERT were measured by real-time RT-PCR and western blot. For real-time RT-PCR, Hep-G2 cells were incubated with EEAL at concentrations of 0, 5, 50 and 200 μg/mL for 48 h. As shown in Figure 4, AL inhibits the mRNA expressions of hTERT and c-myc in a dose-dependent manner. The mRNA expressions of hTERT and c-myc were reduced the most in Hep-G2 cells incubated with

200 μg/mL EEAL, which decreased to 15% and 10%, respectively.

The protein expressions of c-myc and hTERT were also reduced by AL in Hep-G2 cells (Figure 5), which declined with increasing AL concentration. The observations suggest that AL inhibits the expression of c-myc and hTERT to down-regulate the telomerase activity in Hep-G2 cells.

Discussion

In this study, we demonstrated for the first time that AL is able to inhibit proliferation through down-regulation of c-myc/hTERT/telomerase pathway in Hep-G2 cells. We showed that AL induced apoptosis and arrested cell cycle on G1 phase to reduce proliferation.

Atractylis lancea (Thunb.) DC. has been used for the treatment of gastrointestinal diseases and flu for more than thousands years in China. In recent years, AL and its components have been exhibited anti-cancer effect in several cancer cells. The ethanol extract of *Atractylis lancea (Thunb.) DC*. effectively inhibits cell viability in CL-6 and Hep2 cells (Donate & Blasco, 2011). Atractylenolide II and III, the major components of AL induce apoptosis and reduce proliferation in melanoma and lung cancer cells, respectively. In B16 cells, Atractylenolide II arrests cell cycle at G1 phase via down-regulation of CDK2 and activation of caspase-3 and 8 (Ye et al., 2011). Atractylenolide III modulates caspase-3, caspase-9 and bax to induce apoptosis in A549 cells (Kang et al., 2011). In present study, our results that AL inhibits proliferation and arrests cell cycle on G1 phase to induce apoptosis, are accordance with the above mentioned researches.

Telomerase has been considered as an important potential target in the development of anti-cancer therapy and drugs research. hTERT, an essential subunit of telomerase, is directly correlated with telomerase activity. In most cancer, both hTERT expression and telomerase activity are in high level to support proliferation. In recent years, many researches have demonstrated that many plants and its compounds can inhibit tumor proliferation and induce apoptosis via down-regulation of telomerase activity, such as aqueous extract of P. urinaria and the water extract of militaris, which can effectively reduce tumor growth and proliferation via inhibiting telomerase activity and hTERT expression in vitro and in vivo (Park et al., 2009; Huang et al., 2010).

In our research, we showed that the telomerase activity is reduced by AL in dose-dependent manner in Hep-G2 cells. Analyses of hTERT mRNA and protein expressions by real-time RT-PCR and western blot showed that AL reduces the hTERT mRNA and protein levels. These results implied that effect of AL on anti-telomerase activity might result from attenuating the mRNA and protein expression of hTERT.

As a transcription factor, oncogenic protein c-myc has been estimated to regulate up to 15% of human genes which are involved in cell growth and proliferation, apoptosis (Dang, 2012). A number of recent researches indicate that c-myc activation is associated with tumor initiation and progression (Wu et al., 1999). hTERT is a downstream target of c-myc which binds to E-box and promotes expression of the hTERT genes (Henriksson & Luscher, 1996; Cerni, 2000). Therefore, c-myc and hTERT have been considered as the key factors on the anti-telomerase strategy for cancer therapy (Ward & Thompson, 2012; Zhao et al., 2013). In our work, AL shows the inhibitory effect on c-myc mRNA and protein expressions analyzed by real-time RT-PCR and western blot. We speculate that AL reduces telomerase activity through down-regulation of c-myc/ hTERT pathway.

In conclusion, the present study served to confirm the anti-proliferation effect of AL through down-regulation of c-myc/hTERT/telomerase pathway in Hep-G2 cells. Our results enhance the understanding of the anti-tumor mechanism of AL and support the application of AL in clinical treatment of liver cancer.

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