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Blocking inhibition to YAP by ActinomycinD enhances anti-tumor efficacy of Corosolic acid in treating liver cancer

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ABSTRACT

Chemotherapy is critical for the treatment of liver cancer. Despite the pro-apoptotic effects of corosolic acid (CA) have been revealed, the methods to enhance its efficacy are unclear. The aim of this study is to investigate the target that might reduce CA efficacy and figure out the way to conquer it. We found reduction of Yes-associated protein (YAP) might be a critical event that suppresses efficacy of CA. Treatment of CA accelerated degradation of YAP via enhancing its phosphorylation by LATS1. Moreover, we found CA boosts βTrCP-dependent Ubiquitination of YAP. Interestingly, the protein stability of βTrCP per se could be enhanced by CA. Notably, ActionomycinD (AD) strengthened CA-induced apoptosis of liver cancer cells via elevating YAP while down-regulating βTrCP. Importantly, combined treatment of CA and AD had much more obvious influences against transformative phenotypes of liver cancer cells than those under treatment of CA alone. Combined usage of AD successfully reduced IC50 value of CA. In summary, we have first uncovered that suppression of YAP might reduce efficacy of CA to treat liver cancer, combined treatment of AD and CA might solve this problem.

1. Introduction

Liver cancer is one of the leading causes of cancer death worldwide [1], and lack of effective medication is one of the main reasons for its high mortality rate. Despite chemotherapy is considered to be an important approach for liver cancer treatment [2], the efficacy of chemotherapy is often unsatisfactory [3]. Therefore, improvements of chemotherapy against liver cancer become urgently essential.

Traditional Chinese herbal medicine has been widely used clinically and proved to be effective in the treatment of malignant diseases [4–5]. Among these herbal medicines, *Actinidia valvata* Dunn has been shown to have cytotoxic effects against several types of cancer cells, including liver cancer cells [6]. Recently, CA has been isolated from *Actinidia valvata* Dunn, and this compound inhibits occurrence and development of tumor [7–9]. Our previous study has also demonstrated that CA functions as a pro-apoptotic agent against cancer cells [10]. However, it's still unknown how to improve efficacy of CA in the liver cancer cells.

Emerging evidences have demonstrated that YAP, the downstream effector of tumor-suppressor Hippo pathway, contributes to liver tumorigenesis [11]. In our previous studies [12–15], we have also concluded that YAP acts as a central node that connects PKA/CREB, MEK/ERK, PI3K/AKT, p70S6K/mTOR and c-Jun./c-Fos signaling, and thereby consists a complex network that finally maintains transformative phenotypes in liver cancer cells. Due to the reasons that CA and YAP play opposite roles on liver cancer, we hypothesized that the efficacy of CA might be closely associated with YAP. Hence, one aim of this study is to clarify the relationship between CA and YAP.

AD is known to be a potent cytotoxic drug [16], and has been clinically used as an agent for treatment against highly malignant cancers [17–18]. Currently, AD is combined used with other anti-tumor agents to treat malignant tumors [19]. However, whether and how AD enhances efficacy of CA remains elusive.

In this study, we have revealed that down-regulation of YAP is a major event that reduces efficacy of CA in liver cancer cells, and combined treatment of AD can conquer this problem. Together, we have

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identified a new strategy that combined usage of CA and AD might increase efficacy of CA in treating liver cancer.

2. Materials and methods

2.1. Cell culture and vectors

All the liver cancer cell lines including SMMC-7721, SK-Hep1, Bel-7402, Bel-7404 and Huh7 were cultured in DMEM (Hyclone, Beijing, China) supplemented with 10% FBS (Australia origin, Gibco, Carlsbad, CA, USA) at 37 °C in an incubator with 5% CO2. ShRNAs against GFP, YAP, or βTrCP were obtained from our previous study [12,20]. LATS1-sh was purchased from Biolink (Shanghai, China). Plasmids expressing YAP-FLAG, LATS1-FLAG and ßTrCP-FLAG were obtained from our previous studies [14,21-22]. Cells were treated with AD (Abcam, Cambridge, MA, USA, final concentration 1 μg/ml), U0126 (Beyotime, Haimen, China, final concentration 1 μg/ml), Forskolin (Beyotime, final concentration 1 μg/ml), SP600125 (Beyotime, final concentration 5 μg/ml), Methanol (Sanjie, Suzhou, China), CA (Sigma, St. Louis, MO, USA, final concentration 0-160 μM), Cycloheximide (CHX, final concentration 50 μg/ml), TPA (Beyotime, final concentration 1 μg/ml) and PD98059 (Beyotime, final concentration 5 μg/ml), respectively.

2.2. Immunefluorescence (IF) and Western blotting (WB)

For IF, cells were fixed by 4% PFA. Next, cells were incubated with blocking buffer (PBS solution contains 3% FBS, 1% Goat serum and 0.1% Triton X-100) for 2 h at room temperature before incubation with primary antibodies that diluted in blocking buffer at 4 °C overnight. The primary antibodies used in the study were anti-YAP (Abcam #52,771), anti-FLAG (Cell signaling technology (CST), Cambridge, MA, USA, #8146) and anti-ßTrCP (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #sc-390,629). Slides were then incubated with Alexa-Fluor-488 or −555 fluorescent conjugated secondary antibodies (CST, #4408, #4412, #4409 or #4413) for 1 h. After that, slides were mounted with ProLong® Gold anti-fade reagent with DAPI (Molecular Probes, Carlsbad, CA, USA) and observed under Carl Zeiss LSM 800 con-focal microscopic system.

For WB, cells were harvested and lysed in a Western/IP buffer (Beyotime, Haimen, China) supplied with protease inhibitor (Roche Applied Science, Indianapolis, IN, USA). The blots were blocked with 5% non-fat milk in Tris–buffered saline (TBS, pH 7.4) for 1 h and then incubated with primary antibodies which are listed as follows: anti-Caspase substrates (CST, #8698), anti-GAPDH (CST, #5174), anti-YAP (Abcam, #52,771), anti-p-YAP (CST,

#13,619), anti-Ub (CST, #3933), anti-p-LATS1 (CST, #8654), anti-LATS1 (CST, #3477), anti-FLAG (CST, #2368), anti-ßTrCP (CST, #4394), anti-Smurf1 (Santa Cruz Biotechnology, #sc-100,616), anti-p-MTS1/2 (CST, #3681), anti-MTS1 (CST, #3682), anti-MTS2 (CST, #3952) or anti-Smurf2 (Abcam, #53,316). The blots were then washed with TBS and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (CST, #7074 or #7076) and visualized by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

2.3. Quantitative RT-PCR (qPCR)

Total RNA was extracted by a Trizol reagent (Invitrogen, Carlsbad, CA, USA). qPCR was performed on the cDNA using primers specific for ßTrCP mRNA (Forward: 5'TGTGGCCAAAACAAAACTTGCC3' and Reward: 5'ATCTGACTCTGACCACTGCTC3'), YAP mRNA (Forward: 5′ GCAGG TTGGGAGATGGCAAAGAC3' and Reward: 5′ GCTTGTTCCCATCCATCA GGAAG3') and GAPDH mRNA (Forward: 5'ATCATCCCTGCCTCTACTGG3' and Reward: 5'GTCAGGTCCACCACTGACAC3'), respectively. RNA input was normalized to the level of GAPDH. All reactions were carried out using SYBR Green Mix (Takara, Dalian, China). qRT-PCR was carried out using an ABI 7500 real-time PCR System (Carlsbad, CA, USA). Data from qPCR were analyzed by the △Ct method [23].

2.4. Cell proliferation, Caspase3/7 activity and soft agar colony formation assays

These assays were performed as described previously [24–25]. Briefly, the cell proliferation activities were measured by an MTT-based experiment. Caspase 3/7 activities were measured by a Caspase 3/7 luciferase Glo reagent (Promega, Madison, WI, USA). For anchorage-independent soft-agar colony formation assay, cells under different treatments were seeded onto six-well plates at a density of 5 × 10^3 cells per well and maintained for further treatments until foci were evident. Colonies were then counted.

2.5. Co-immunoprecipitation (co-IP)

Co-IP was performed as described previously [22]. The reagents used were protein A/G-Sepharose (Novex, Oslo, Norway) and Western/IP lysis buffer (Beyotime) with 0.1% SDS (used for denatured IP for detecting Ubiquitination) or without SDS (used for conventional IP). The antibodies used for IP were: anti-ßTrCP (CST, #4394 or Santa Cruz Biotechnology, #sc-390,629) or anti-YAP (Abcam, #52,771).

2.6. Mice experiments

Bel-7402 cells (5 × 10^6 cells) were subcutaneously injected into 8-week-old athymic nude mice (Bikai, Shanghai, China). After xenografts were visible (24th day after injection), mice were treated with CA (10 mg/kg) with or without AD (0.02 mg/kg) for another 18 days before tumor sizes were measured. The tumor volume was calculated as 0.5 × L × W^2, where L is length and W is width. All mouse experiments were performed according to the institution guidelines of Shanghai Tenth People's Hospital.

2.7. Statistical analysis

Tests to examine the differences between groups included the Student *t*-test and one-way ANOVA. A $p < 0.05$ was regarded as statistically significant. All results are expressed as the means ± standard deviation (SD) of three independent experiments.

3. Results

3.1. Determination of the IC50 value of CA

To determine the IC50 value of CA, we treated Bel-7402 and Bel-7404 cells with a serial concentrations of CA. We found when treated cells with CA at a concentration of 40 μM, the cell proliferation activities reduced to a level of 50% of that from the cells without treatment (Fig. 1A), suggesting 40 μM is the IC50 value of CA.

To further verify the accuracy of IC50, we tested Caspase 3/7 activities and Caspase substrates levels. We found treating cells with a 40 μM CA led to a significant induction of apoptosis, and this level was almost a 50% level of the peak that resulted from a 80 μM CA (Fig. 1B-D). Moreover, we treated cells with CA at the IC50 value, and found it can induce a significant elevation of Caspase substrates level in the entire liver cancer cell lines tested (Fig. 1E). Also, we found a 40 μM of CA could reduce colony formation capacity and cell viability to a 50% level of the cells without CA treatment (Fig. 1F-H). These results demonstrated that CA has an IC50 value of 40 μM when treated as an anti-tumor agent to liver cancer cells.