Autophagic flux is essential for the downregulation of *D*-dopachrome tautomerase by atractylenolide I to ameliorate intestinal adenoma formation

Lu Li¹ · Linlin Jing² · Junjiang Wang³ · Wenjuan Xu⁴ · Xianling Gong^{1,5} · Yiye Zhao² · Ye Ma¹ · Xueqing Yao³ · Xuegang Sun^{1,2}

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Abstract

Colorectal cancer is generally believed to progress through an adenoma - carcinoma sequence. Adenomatous polyposis coli (APC) mutations serve as the initiating event in adenoma formation. The *Apc*^{Min/+} mouse harbors a mutation in the APC gene, which is similar or identical to the mutation found in individuals with familial adenomatous polyposis and 70% of all sporadic CRC cases. Autophagy is a constitutive process required for proper cellular homeostasis. However, its role in intestinal adenoma formation is still controversial. Atractylenolide I (AT1) is a sesquiterpenoid that possesses various clinically relevant properties such as anti-tumor and anti-inflammatory activities. The role of AT1 on adenoma formation was tested in *Apc*^{Min/+} mice and its underlying mechanism in regulating autophagy was documented. *D*-dopachrome tautomerase (*D*-DT) was identified as a potential target of AT1 by a proteomics-based approach. The effects of p53 modification on autophagic flux was monitored in *p53*^{-/-} and *p53*^{+/+} HCT116 cells. Small interfering RNA was used to investigate the function of Atg7 and *D*-DT on autophagy programme induced by AT1. AT1 effectively reduced the formation of adenoma and downregulated the tumorigenic proteins in *Apc*^{Min/+} mice. Importantly, AT1 stimulated autophagic flux through downregulating acetylation of p53. Activation of Sirt1 by AT1 was essential for the deacetylation of p53 and downregulation of *D*-DT. The lowered expression of COX-2 and β -catenin by AT1 were partly recovered by Atg7 knockdown. AT1 activates autophagy machinery to downregulate *D*-DT and reduce intestinal adenoma formation. This discovery provides evidence *in vivo* and *in vitro* that inducing autophagy by natural products maybe a potential therapy to ameliorate colorectal adenoma formation.

Lu Li, Linlin Jing and Junjiang Wang contributed equally to this work.

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✉ Xuegang Sun
sxxg_smu@126.com

Lu Li
549812447@qq.com

Linlin Jing
jll47379@163.com

Junjiang Wang
langyisheng6726582@qq.com

Wenjuan Xu
xwjydc@163.com

Xianling Gong
1290064145@qq.com

Yiye Zhao
1056882895@qq.com

Ye Ma
915242251@qq.com

Xueqing Yao
yjb9211@21cn.com

- ¹ School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, China
- ² TCM Integrated Hospital of Southern Medical University, Guangzhou 510315, China
- ³ Department of Gastrointestinal Surgery, Guangdong General Hospital, Guangzhou 510120, China
- ⁴ School of Traditional Chinese Medicine, Binzhou Medical University, Yantai, Shandong 256600, China
- ⁵ School of Pharmacy, Guangdong Medical University, Dongguan, Guangdong 523808, China

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Background

The adenoma–carcinoma sequence represents the process by which most, if not all, colorectal cancer (CRC) arise. The normal colon epithelium starts with constitutive activation of the Wnt signaling, followed by adenoma formation and ends with adenocarcinoma transformation and invasion (Sun et al. 2016). Although mildly declined in the past decade, it remains the third most common cancer and accounts for about 8% cancer deaths in American in 2017 (Siegel et al. 2017). The poor overall survival of CRC underscores the rationale to elucidate causes and to search for preventative approaches to interfere the malignant sequence (Siegel et al. 2017).

Majority of colorectal carcinomas (60%) and adenomas (63%) contained a mutated adenomatous polyposis coli (APC) gene (Powell et al. 1992). Loss of APC function constitutively activates the formation of stable β -catenin/T cell transcription factor-4 complex which is a crucial event in the early transformation of colonic epithelium (Korinek et al. 1997). The $APC^{Min/+}$ mouse harbors a heterozygous, dominant mutation in the APC gene and, like individuals with familial adenomatous polyposis, develop intestinal adenomatous polyps that progress to carcinomas. So, $APC^{Min/+}$ mouse is a valuable research tool for for analysis and prevention of human FAP and sporadic colorectal cancers (Chang et al. 2017).

Autophagy (specifically macroautophagy) is an evolutionarily conserved catabolic process where the cytoplasmic contents of a cell are sequestered within double membrane vacuoles (Cruz and Brekken 2017; Chen et al. 2017). However, studies of the relationship between autophagy and adenoma formation or carcinogenesis have produced contradictory results. For example, conditional deletion of Atg7 in the intestinal epithelium did not increase the number of intestinal polyps (Nishiumi et al. 2012). On the other hand, heterozygous deletion of Atg5 in $Apc^{Min/+}$ mice increased the number and size of adenomas through activation of EGFR/ERK1/2 and Wnt/ β -catenin pathways (Wang et al. 2015). These controversial results suggest that further investigation is needed to clarify the role of autophagic flux in the formation of intestinal adenoma.

AT1, a major sesquiterpenoid of *Atractylodes macrocephala*, significantly decreased viability of lung carcinoma cell lines A549 and HCC827 through inducing intrinsic apoptosis (Liu et al. 2013). It inhibited bladder cancer cell proliferation and arrested cell cycle in G2/M phase by inhibition of PI3K/Akt/mTOR signaling pathway (Yu et al. 2016). It also sensitized human epithelial ovarian carcinoma cells to paclitaxel by inhibiting TLR4/MyD88-dependent pathway (Huang et al.

2014). We thus proposed that AT1 might ameliorate tumorigenesis through disturbing the adenoma - carcinoma sequence. To test the hypotheses, $APC^{Min/+}$ mice were harnessed to test the effects of AT1 on carcinogenesis. Our results indicated that the AT1 stimulated autophagic flux to downregulate expression of *D*-dopachrome tautomerase (*D*-DT), which is a crucial upstream activating cytokine that regulated intestinal adenoma formation. Interestingly, with $p53^{+/+}$ (wild type) and $p53^{-/-}$ HCT116 cells, we found that deacetylation of p53 was pivotal for the induction of autophagic flux. These results suggested that AT1 ameliorates intestinal adenoma formation through autophagy mediated *D*-DT suppression.

Methods

Compounds and reagents MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Atractylenolide-I, (4aS,8aS)-3,8a-dimethyl-5-methylidene-4a,6,7,8-tetrahydro-4H-benzo[f] benzofuran-2-one (MF C15H18O2, MW (g/mol) 230.3022, HPLC $\geq 98\%$) was purchased from Chenguang-Reagent Co. Ltd. (Baoji, Shaanxi, China); the rabbit monoclonal antibodies to p53, acetylated p53, phospho-p53 antibodies were bought from CST (Cell Signaling Technology, MA, USA) and Abcam (Cambridge, United Kingdom). horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) (Supplementary material, Table S1).

Animals $APC^{Min/+}$ male mice on a C57BL/6 background (purchased from Jackson Laboratories, PA, USA) were bred with female C57BL/6 mice in Southern Medical University. C57BL/6 J mice (specific pathogen-free) were obtained from the Laboratory Animal Center of Southern Medical University. Offsprings were genotyped as heterozygotes by real-time polymerase chain reaction (RT-PCR) for the APC gene by taking tail snips at weaning (Supplementary material, Figure S1, Table S2). Six C57BL/6 mice of SPF grade (3 males and 3 females), 4-weeks-old, weighing (20 ± 2) g, were used as a wild-type control. Mice were maintained on a 12:12 h light–dark cycle in a low-stress environment and provided food and water ad libitum. All procedures involving laboratory animals were in accordance with the guidelines of the Instituted Animal Care and Use Committee of Southern Medical University. All protocols were submitted and validated by the Animal Care Ethics Committee of Southern Medical University.

Cell lines and culture Human Colon cell lines HCT116 was obtained from American Type Culture Collection (ATCC, Rockville, MD). HCT116 *p53*^{-/-} was gift from Prof. Zhiling Yu, Hong Kong Baptist University. All cell lines were incubated in RPMI-1640 (Invitrogen) and supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1% (v/v) penicillin–streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂.

Proteomic analysis Aliquots of tissue were homogenized in liquid nitrogen and resuspended in lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM β-glycerophosphate, 250 mM NaCl, 2% NP-40 and protease inhibitors). Protein extraction, 2-DE, Gel Staining, Tryptic Digestion of 2-DE Gel Spots and Mass Spectrometry (MS) identification were performed as previously described according to standard protocols (Chunhua et al. 2013). Gel images were analyzed by Melanie 6.0. Reference points were defined in the center of the gels.

Quantitative real-time PCR Total RNA was extracted with TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed with cDNA synthesis kit (TaKaRa, Dalian, China). Quantitative real-time PCR was performed using the SYBR Green real-time PCR kit (TaKaRa) (Supplementary information, Table S2). The fold changes were calculated by the delta–delta Ct method. All experiments were performed in three biological replicates.

siRNA synthesis and transfection Three siRNAs for *D*-DT were designed by Ambion's siRNA Target Finder. The siRNAs for *D*-DT, Atg7 (Supplementary material, Table S3) and control siRNAs were synthesized by Shanghai GenePharma (Zhangjiang Hi-Tech Park, Shanghai, China). The siRNAs were transfected with Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instruction as previously described (Chunhua et al. 2013). The transfection rates were measured using fluorescence microscopy.

Immunoblotting analysis Immunoblotting was performed as described previously (Sun et al. 2015). Briefly, 30 μg of protein lysates from blank cells and cells exposed to AT1 were boiled in SDS-PAGE gel loading buffer, subjected to SDS-PAGE, transferred to PVDF membrane at 100 V for 100 min at 4 °C, and incubated with the respective primary antibody at 4 °C overnight and then incubated with the horseradish peroxidase-conjugated secondary antibody. The optical density was normalized against that of β-actin (Chunhua et al. 2013).

Immunofluorescence HCT-116 cells were incubated with antibody LC3-II (Cell Signaling) at 4 °C overnight, incubated with Cy3-labeled goat anti-rabbit or anti-mouse IgG antibody (1:500) in darkness for 60 mins at room temperature, and then counterstained with 4',6-diamidino-2-phenylindole (DAPI), and examined under confocal microscope (Nikon, Japan) with excitation and emission wavelengths of 550 and 570 nm, respectively with a 100 × 1.40 NA oil immersion objective (Xu et al. 2015).

Immunohistochemistry Immunohistochemistry was performed as described previously (Sun et al. 2015). Paraffin-embedded colon sections were dewaxed, rehydrated, and pre-treated with hydrogen peroxidase in PBS buffer. Heat-induced antigen retrieval was performed. After blocking with the appropriate antisera in blocking buffer, sections were incubated with anti-Proliferating cell nuclear antigen PCNA, β-Catenin, COX-2. After incubation with HRP conjugated secondary antibody and tyramide amplification followed by streptavidin-HRP, positive signals were visualized by DAB kit, and counter-stained with hematoxylin. Five randomly selected fields from each section were examined at a magnification of 400× and analyzed using NIS-Elements. The positive content was calculated using the following formula: positive content (PC) = mean optical density × positive area.

The immunohistochemical staining results were assigned a mean score considering both the intensity of staining and the proportion of tumor cells with an unequivocal positive reaction. Each section was independently assessed by 3 pathologists without prior knowledge of patient data.

Positive reactions were defined as those showing yellow to brown signals in the cell cytoplasm. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The frequency of positive cells was defined as follows: 0, less than 5%; 1, 5 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, greater than 75%. Five fields were chosen from each immunohistochemical section, each field was analyzed by microscope (400×). For statistical analysis, scores of 0 is negative (–), scores of 1 to 4 is weak (+), scores of 5 to 8 is moderate (++), scores of 9 to 12 considers high expression (+++).

Statistical analysis Data are expressed as the mean ± standard deviation (SD) for each experimental group. All the data were analyzed using the SPSS statistical package (SPSS, version 17.0; SPSS, Inc.). Univariate analysis was performed using one-way ANOVA, factorial analysis. The differences between two groups were analyzed, based on the test of least significance difference (LSD). If the variances were not homogenous, mean values were compared using Welch's test. The differences between two groups were analyzed by Dunnett's T3. A *p* value of *P* < 0.05 was considered statistically significant.

Availability of data and materials All data generated during this study are included in this published article and its Additional files.

Results

AT1 reduces adenoma formation in *Apc^{Min/+}* mice

To investigate if AT1 could prevent carcinogenesis, *Apc^{Min/+}* mice treated with AT1 were chosen to observe the formation of intestinal adenoma. All intestinal polyps in *Apc^{Min/+}* mice were histologically identified as adenoma. AT1 reduced the number of adenoma significantly at the dose of 25 and 50 mg/d compared to the *Apc^{Min/+}* mice (Fig. 1a–b).

The expressions of PCNA, β -catenin, and COX-2 were elevated in adenoma tissues of *Apc^{Min/+}* mice. AT1 treatment decreased their expression significantly (Fig. 1c–d). As the upregulated β -catenin and PCNA are required for tumor development in the colon, and increased COX-2 enhanced tumorigenesis, our results suggested that AT1 downregulated

the tumorigenic proteins and reduced the formation of adenoma in *Apc^{Min/+}* mice effectively.

D-DT is a potential target of AT1

To identify the target responsible for the reduced adenoma, a proteomics-based approach was adopted to distinguish differentially expressed proteins response to AT1 (25 mg/kg/d) treatment in *Apc^{Min/+}* mice (Fig. 2a). Among the 22 proteins that successfully identified (Supplementary material, Table S4), D-DT (point X8) excited our interest for its role in inflammation and carcinogenesis (Xin et al. 2010). D-DT is a member of the macrophage migration inhibitory factor (MIF) protein superfamily that regulates both the innate and the adaptive immune response. D-DT binds the MIF cell surface receptor complex, CD74/CD44, with high affinity and induces similar cell signaling and effector functions (Merk et al. 2012). So, it is proposed to designate as “MIF-2” (Merk et al. 2012). We examined the expression of both MIF-1 and D-DT in AT1 treated adenoma tissues. AT1 reduced the expression of MIF-1 and D-DT significantly and it

Fig. 1 Atractylenolide I reduces adenoma formation **a** Microscopic view of colon in mice. **b** Effect of atractylenolide I (AT1) on the adenoma number in *Apc^{Min/+}* mice ($n = 6$). Data were presented as mean $\pm$ SD. * $P < 0.05$ vs control, ** $P < 0.05$ vs vehicle. **c** Immunohistochemical staining of β -catenin, COX-2 and PCNA expression in adenoma tissues

(*Apc^{Min/+}* mice) and normal colonic tissues (C57BL/6 J) and AT1 treated mice. 400 $\times$ for all, scale bar = 20 μ m. **d** Scores of the Immunohistochemical (c) use the standard for scores as we mentioned before. Data were presented as mean $\pm$ SD. * $P < 0.01$ vs control, ** $P < 0.01$ vs vehicle

Fig. 2 Proteomic identification of *D*-DT as a potential target of AT1
a Representative two dimensional gel electrophoresis (2DE) gels showing the locations of spots with different intensities in Model and Atractylenolide I groups are shown. Protein extraction, 2-DE, and gel staining were performed as previously described. **b** Western blot analysis the expression of MIF-1, *D*-DT, COX-2 and β -catenin in control colonic tissues (C57BL/6 J) and adenoma (*Apc*^{Min/+} mice) treated with or without AT1

decreased the expression of COX-2 and β -catenin, either (Fig. 2b). Collectively, *D*-DT might be a potential target for AT1 to regulate the tumorigenic protein and adenoma formation.

AT1 induces the formation of autophagosome

It has been demonstrated Atg16L1-deficient macrophages showed impaired autophagosome formation and degradation of long-lived proteins and produced high amounts of inflammatory cytokines IL-1 β and IL-18 (Saitoh et al. 2008). Loss of autophagy enhanced MIF secretion by monocytes and macrophages which depends on mitochondrial reactive oxygen species (ROS) (Lee et al. 2016). We hypothesized that the decreased *D*-DT and MIF-1 by AT1 might be caused by the promotion of autophagy in *Apc*^{Min/+} mice. As expected, AT1 treatment increased the expression of Atg5, Atg7, and Beclin. It also promoted the conversion of LC3I to LC3II through proteolytic cleavage and lipidation in intestinal tissues of *Apc*^{Min/+} mice (Fig. 3a). We then verified the effects of AT1 on autophagy in colon carcinoma cell line HCT116. AT1 increased the expression of Atg7, Beclin and LC3B in a dose-dependent manner (Fig. 3b). Immunofluorescent staining provided direct evidences that AT1 induced the punctae of LC3-II around the nucleus (Fig. 3c). To further confirm the formation of autophagosome, transmission electron microscopy showed that double-membrane vacuoles were increased by AT1 significantly (Fig. 3d–e). Taken together, these results demonstrated that AT1 stimulated the formation of autophagosome both *in vitro* and *in vivo*.

AT1 activates Sirt1 and deacetylates p53

Mammals have seven silent information regulator (Sir)2 homologs (sirtuins, Sirt1–7) that regulate oxidative stress, DNA damage, and metabolism, et al. Sirt1 is an important *in vivo* regulator of autophagy. It is also a deacetylase that deacetylates p53 tumor suppressor protein (Kim et al. 2007; Chang et al. 2015). We first examined the effect of AT1 on the expression of Sirt1 in HCT116 derived *p53*^{+/+} (wild type) and *p53*^{-/-} cells. Quantitative PCR showed that AT1 significantly increased the expression of Sirt1 in HCT116 *p53*^{+/+} cells while Sirt1 level was mildly reduced in HCT116 *p53*^{-/-} cells (Fig. 4a). Next, we checked the protein expression of Sirt1 in two cell lines. As expected, AT1 increased the expression of Sirt1 in a dose-dependent manner in HCT116 *p53*^{+/+} cells while it had no effect in HCT116 *p53*^{-/-} cells (Fig. 4b). The expression of total p53 and phospho-p53 was not significantly changed in both cell lines with or without AT1 treatment. As p53 is the most widely known substrate of Sirt1, the acetylation statues of p53 after AT1 treatment was assessed and significantly reduced acetylation of p53 levels was observed. As deacetylation of p53 induces autophagy flux (Contreras et al. 2013). AT1 may stimulate autophagy through downregulating acetylation of p53.

EX-527 is a specific inhibitor of SIRT1 activity (Supplementary material, Figure S2). It significantly reduced expression of Sirt1 (Fig. 4c) and increased the acetylation of p53 (Supplementary material, Figure S2). It suppressed the expression of Atg5 and Atg7 and lowered the conversion of LC3I to LC3II after AT1 treatment (Fig. 4c). Collectively, AT1 induces autophagy through deacetylation of p53 in a Sirt1 dependent manner.

Fig. 3 Atractylenolide I induces autophagic flux **a** Protein expression levels of autophagy in control colonic tissues (C57BL/6 J) and adenoma (*Apc^{Min/+}* mice) treated with or without AT1, ($n=3$). **b** Western blot of Atg7, Beclin-1 and LC3-II expression on HCT116 cells exposed to 0, 25, 50, 80, 100 μM AT1, ($n=3$). **c** Immunofluorescent

staining analysis the distribution of LC3-II punctae (Red arrowhead), ($n=3$). **d** Autophagosomes changes of AT1-treated cells as observed by TEM (Red arrowhead). 10,000 $\times$ for all, scale bar = 0.5 μm , ($n=3$). **e** Statistical analysis of autophagosomes in (**d**), ** $P<0.05$ vs AT1 0 (μM), ($n=3$)

AT1 induces autophagy to downregulate D-DT

The relationship between autophagy and MIF-1, D-DT was checked in HCT116 cells treated with AT1 from 0 μM to 100 μM . The upregulated expression of LC3II suggested the induction of autophagy. AT1 also reduced expression of COX-2 and β -catenin (Fig. 5a).

To further elucidate the role of autophagy in the regulation of D-DT and carcinogenesis, chloroquine, an antimalarial lysosomotropic agent which inhibiting lysosome-autophagosome fusion and lysosomal protein degradation was adopted. Chloroquine increased the level of D-DT and β -catenin and increased acetylation of p53, suggested that the inhibition autophagy promoted accumulation of D-DT and β -catenin. These results suggested that autophagy is essential for the downregulation of D-DT (Fig. 5b).

Bafilomycin A1, a member of the plecomacrolide subclass of macrolide antibiotics, is a specific inhibitor of the vacuolar type H^+ -ATPase in cells. It increased the expression of LC3-II through preventing the maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. The disruption of autophagy process subsequently inhibited the effects of AT1 on lowering the expression of

COX-2 and β -catenin (Fig. 5c). Together, AT1 downregulates D-DT through p53 mediated autophagy induction.

AT1 induced autophagosome formation is essential for downregulation of carcinogenesis proteins

To explore the role of autophagosome formation in regulation of carcinogenesis by AT1, Atg7 was knockdown by siRNA. Atg7 knockdown inhibited the formation of autophagosome by lowering LC3-II. The lowered expression of carcinogenesis markers, such as COX-2 and β -catenin were partly recovered by Atg7 siRNA. The expression of D-DT was also elevated by the knockdown of Atg7 (Fig. 6a).

The downregulation of COX-2 and β -catenin, suggesting that D-DT is at least partially responsible for the regulation of carcinogenesis (Supplementary material, Figure S3). AT1 further reduced the expression of COX-2 and β -catenin also indicated that other mechanisms might be involved in the regulation of carcinogenesis by AT1 (Fig. 6b). Collectively, our results show that autophagy induced downregulation of D-DT is at least partially responsible for the regulation of adenoma formation by AT1 (Supplementary material, Figure S4).

Fig. 4 AT1 induces Sirt1 and deacetylates p53 **a** mRNA expression levels of Sirt1 in HCT116 *p53*^{+/+} cells and HCT116 *p53*^{-/-} cells. Data were presented as mean ± SD. **P* < 0.05 vs AT1 0 (μM), ***P* < 0.01 vs AT1 0 (μM), (*n* = 3). **b** Protein expression levels of Sirt1, p53, P-p53 and Ac-

p53 in HCT116 *p53*^{+/+} cells and HCT116 *p53*^{-/-} cells exposed to 0, 25, 50, 80, 100 μM AT1, (*n* = 3). **c** Protein expression levels of autophagy in HCT116 cells exposed to Ex527 (1 μM) and AT1 (100 μM), (*n* = 3)

Fig. 5 Autophagy induced downregulation of *D*-DT is at least partially responsible for the regulation of adenoma formation by AT1 **a** Protein expression levels of MIF-1, *D*-DT, COX-2 and LC3-II in HCT116 cells treated with AT1 (100 μM), (*n* = 3). **b** Protein expression levels Ac-p53,

p53 and MIF-1 in HCT116 cells treated with AT1 (100 μM) and CQ (10 μM), (*n* = 3). **c** Protein expression levels *D*-DT, COX-2 and β-catenin in HCT116 cells after treated with Baf A1 (100 nM) and AT1 (100 μM), (*n* = 3)

Fig. 6 AT1 downregulates D-DT by inducing autophagosome formation **a** Protein expression levels LC3-II, D-DT, COX-2, β -catenin and Ac-p53 in HCT116 cells after transfected with Atg7 siRNA and treated with AT1 (100 μ M), ($n = 3$). **b** Protein expression levels D-DT, COX-2 and β -catenin in HCT116 cells after transfected with D-DT siRNA and treated with AT1 (100 μ M), ($n = 3$)

Discussion

D-DT is a novel hypoxia-inducible gene and a direct target of HIF-1 α and HIF-2 α (Pasupuleti et al. 2014). It is the only known homolog of MIF that cooperatively signaling with MIF-1 in a CD74-dependent manner. It is highly expressed in CRC, non-small cell lung carcinomas (Coleman et al. 2008) clear cell renal cell carcinomas (Pasupuleti et al. 2014) and pancreatic ductal adenocarcinoma (Guo et al. 2016). D-DT is found to work cooperatively with MIF-1 to antagonize the tumor suppressor pathways associated with activated AMP-activated protein kinase (AMPK) and tumor suppressor p53 (Brock et al. 2012; Brock et al. 2014). D-DT and MIF-1 compensate for each other to induce angiogenic growth factor CXCL8 and VEGF expression through JNK activation (Coleman et al. 2008). In our report, both D-DT and MIF-1 were activated in the intestinal adenoma with high expression of COX-2 and β -catenin (Xin et al. 2010). Knockdown of D-DT decreased the expression of these oncogenic proteins, suggesting that D-DT is an important regulator in the formation of intestinal adenoma.

The role of p53 in autophagy is context-dependent. Nuclear p53 promotes autophagy, while cytoplasmic p53 inhibits it (Green and Kroemer 2009). Inhibition of p53 can induce autophagy in nematodes and mammalian cells, and cytoplasmic p53 is mainly responsible for this autophagy inhibition (Ranjan and Iwakuma 2016). HCT116 cells with or without wild type p53 were treated with AT1, however, no significant inhibition was observed in our results. Recently it has been reported that deacetylation and nuclear accumulation of p53 suppress Bmf expression and thereby facilitate autophagy

(Contreras et al. 2013). We thus investigated if posttranslational modification was regulated by AT1. The acetylation was found to be reduced in a dose dependent manner while the phosphorylation level was not changed. Deacetylation level of p53 was negatively correlated to the expression of Sirt1, demonstrating that Sirt1 might upregulate autophagy through p53 deacetylation (Contreras et al. 2013; Ou et al. 2014).

Sirt1 is a NAD⁺-dependent deacetylase that can induce autophagy (Li et al. 2016). After inhibiting Sirt1 with Ex527, the autophagic flux was downregulated even in the presence of AT1. CQ is a 4-aminoquinoline drug that inhibits lysosomal acidification and therefore prevents autophagy by blocking autophagosome fusion and degradation (Jaraúta et al. 2016; Gewirtz 2016). CQ treatment partly regains the acetylation of p53, accompany with the upregulation of D-DT and COX-2, indicated that autophagy is the key mechanism in regulating intestinal adenoma formation.

Our previous work showed that Shenling Baizhu San decreases the incidence and multiplicity of azoxymethane and dextran sodium sulfate induced colitis associated CRC (Lin et al. 2015). AT1 is a representative ingredient of *Rhizoma Atractylodis Macrocephalae*, which is the most widely prescribed Chinese herb in prevention and treatment of malignant tumors (Lin et al. 2015). Autophagy is a constitutive process required for proper cellular homeostasis (Rioux et al. 2007). Autophagic digestion of endogenously synthesized cytosolic proteins enables their processing for MHC II presentation which is important for the immune elimination of transformed cells (Sun et al. 2016; Wang et al. 2017). Collectively, our results provide evidence that AT1 elevates autophagic flux to maintain homeostasis and eliminate cancerous cells. It

might ameliorate the inflammatory microenvironment by decreasing *D*-DT to interrupt the progression of adenoma in *APC^{min/+}* mice.

Conclusion

These results put together suggest that AT1 reduces intestinal adenoma formation through activating autophagy machinery to downregulate *D*-DT. This discovery provides evidence *in vivo* and *in vitro* that inducing autophagy by natural products is a safe and effective therapeutic strategy to ameliorate adenoma formation.

Authors' contributions LL carried out the experiments; LJ, JZ, WX and XG helped to carried out the experiments; YZ and YM carried out statistical analyzes; XY helped in designing few experiments; XG designed the experiment and wrote the manuscript; All the authors reviewed the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Ethical approval and consent to participate All procedures involving laboratory animals were consented by the Instituted Animal Care and Use Committee of Southern Medical University.

Consent for publication Not Applicable.

Competing interests All authors declare that they have no competing interests.

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