Chinese Journal of Integrative Medicine
Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Original Article

Gambogic Acid Induces Cell Apoptosis and Inhibits MAPK Pathway in PTEN$^{-/-}$/p53$^{-/-}$ Prostate Cancer Cells *In Vitro* and *Ex Vivo**

PAN Hong[1,2,3], LU Li-yuan[2], WANG Xue-qian[2], LI Bin-xue[2], Kathleen Kelly[3], and LIN Hong-sheng[2]

ABSTRACT **Objective:** To investigate the effect of gambogic acid (GA) on the growth and cell death of castrate resistant prostate cancer (PC) with phosphate and tension homology (PTEN) and p53 genes deleted *in vitro* and *ex vivo*, and elucidate the underlying possible molecular mechanisms. **Methods:** PTEN$^{-/-}$/p53$^{-/-}$ PC cells and Los Angeles prostate cancer-4 (LAPC-4) cells were treated with GA for 24 h and 48 h, then cell viability was determined by cell proliferation assay. PTEN$^{-/-}$/p53$^{-/-}$ PC cells organoids number was calculated under GA treatment for 1 week. In addition, cell titer glo assay was performed to analyze 3 dimensional cell viability of patients derived xenografts (PDX) 170.2 organoids. Flow cytometry was used to detect apoptotic cells treated with GA. And confocal image was performed to detect the apoptotic mitochondrial morphological changes. Apoptotic cell death related protein levels were measured through Western blot (WB) in GA treated cells and organoids. The expression levels of mitogen-activated protein kinases (MAPKs) pathway related ribonucleic acid (RNAs) and proteins were analyzed by reverse transcription polymerase chain reaction (RT-PCR) and WB, respectively. **Results:** The treatment of GA significantly reduced cell viability of PTEN$^{-/-}$/p53$^{-/-}$ PC cells and LAPC-4 in a time- and concentration-dependent manner. In organoids, GA showed strong inhibition towards organoids' numbers and diameters and continuously led to a complete organoids inhibition with GA 150 nmol/L. *Ex vivo* results validated that GA 1 μmol/L inhibited 44.6% PDX170.2 organoids growth. As for mechanism, flow cytometry detected continuously increased apoptotic portion under GA treatment from 1.98% to 11.78% (6 h) and 29.94% (8 h, P<0.05). In addition, mitochondrial fragmentation emerged in GA treated cells indicated the mitochondrial apoptotic pathway might be involved. Furthermore, WB detected caspases-3, -9 activation and light chain (LC)-3 conversion with GA treatment. WB revealed decreased activity of MAPK pathway and down-regulation of downstream c-fos oncogene RNA level was detected by RT-PCR before undergoing apoptosis (P<0.05). **Conclusion:** GA was a potent anti-tumor compound as for PTEN$^{-/-}$/p53$^{-/-}$ PC, which contributed to cell apoptosis via inhibition of the MAPK pathway and c-fos.

KEYWORDS gambogic acid, prostate cancer, apoptosis, mitogen-activated protein kinase, PTEN$^{-/-}$/p53$^{-/-}$

Prostate cancer (PC) is the most common and the second leading cause of cancer death in the Western male, with estimated 238,590 new cases and 29,720 deaths in 2013.[1] GLOBOCAN 2012 data found that the incidence of PC among East Asian male is 10.5 per 100,000 men and that mortality is 3.1 deaths per 100,000 men, which is significantly lower than incidence and mortality rates among Western male.[2] Studies evaluating PC in Chinese male have previously suggested that the prevalence of PC is between 3% and 20%.[3] However, a recent study of 340 Chinese cystoprostatectomy specimens revealed that 28% of specimens had incidental PC identified, suggesting that the true prevalence of PC in Chinese male is significantly higher than previously recognized and perhaps comparable to that of Western populations.[4]

*Supported by an International Science and Technology Cooperation Program (No. 2013DFA32540), China
1. Clinical Medical College, Beijing University of Chinese Medicine, Beijing (100029), China; 2. Laboratory of Oncology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing (100053), China; 3. Laboratory of Genitourinary Cancer Pathogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD (20814), USA
Correspondence to: Prof. LIN Hong-sheng, Tel: 86-10-88001192, E-mail: drlinhongsheng@163.com
DOI: https://doi.org/10.1007/s11655-017-2410-3

Chin J Integr Med 2018 Feb;24(2):109-116

PC displays a range of clinical behavior from relatively slow-growing tumors to aggressive disease with poor prognosis with 10%.[5] Phosphate and tension homology (PTEN) tumor suppressor loss occurs in >70% of PCs, and biallelic deletion of PTEN is correlated with castrate resistant in PC.[6] Large scale studies have shown that p53 mutations occur in 26% castrate resistant PC.[7,8] All the result demonstrates that abnormal PTEN and p53 contributes to PC progression and castrate resistant form, which remain a challenge in PC treatment.

Gambogic acid (GA) is a major active ingredient extracted from Chinese herbal medicine, *Garcinia gamboge hanburyi*,[9] which has a long history of use for detoxification, homeostasis, anti-inflammatory and parasiticide medicines.[10] The molecular structure of GA was shown in Figure 1. GA was initially reported as an effective apoptosis inducer through high-throughput screening,[11] the function of which has been validated towards various tumor cells, such as lung, breast and PCs.[12-15] Though, emerging evidences had demonstrated GA as a potent apoptosis inducer, the role of which in castrate resistant PCs were barely investigated and the underlying mechanism needed to be fully elucidated.

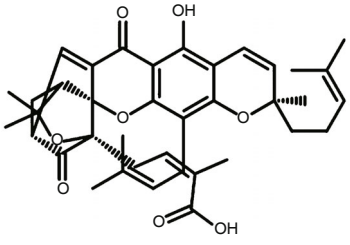

Figure 1. Chemical Structure of GA ($C_{38}H_{44}O_8$, molecular weight: 628.75 g/mol)

In the present experiment, we determined the anti-tumor effect of GA in castrate resistant PC in not only organoids culture as for *in vitro*, but also a castrate resistant patient derived xenograftes (PDX) model as for *ex vivo*. Besides, underlying molecular pathway of GA induced growth inhibition was partially revealed.

METHODS

Chemicals and Reagents

GA was purchased from National Institute for Food and Drug control (Beijing, China) and dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 5 mmol/L and stored protecting from light in –20 ℃ for future use within 2 months. WIT-P medium was purchased from Cellaria Biosciences (Cambridge, MA, USA). Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), trypsin and penicillin-streptomycin solution were obtained from Gibco (Waltham, MA, USA). Cell Titer 96 AQ_{ueous} one solution cell proliferation assay kit and Cell Titer-Glo® (CTG) 3 dimensional (3D) cell viability assay kit were purchased from Promega (Madison, WI, USA). Matrigel™ matrix with growth factor reduced or not were purchased from Corning (Corning, NY, USA). Propidium iodide (PI), Hoechst 33342, MitoTracker® Deep Red FM and West Femto substrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies of caspase-3, -9, light chain 3 (LC-3); total mitogen-activated protein kinase kinase1/2 (T-MEK1/2), phospho-MEK1/2 (P-MEK1/2), total mitogen-activated protein kinase1/2 (T-ERK1/2), P-ERK1/2, P-S6 were obtained from Cell Signaling Technology (Boston, MA, USA). Primary antibodies of α-tublin, protease inhibitor, DMSO, triton X-100 and polysorbate 20 (known as tween 20) were bought from Sigma (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer and phosphatase inhibitor cocktail were purchased from Boston Bio-products (Ashland, MA, USA). Mini-PROTEAN TGX precast gels and polyvinylidene fluoride (PVDF) transfer membrane were purchased from Bio-Rad (Hercules, CA, USA). RNeasy mini kit was purchases from Qiagen (Hilden, Germany).

2D Cell Culture

$PTEN^{-/-}/p53^{-/-}$ PC cells were originally derived from a prostate adenocarcinoma of genetic engineered mice: $Pb\text{-}Cre^{4+}$; $PTEN^{fl/fl}$; $p53^{fl/fl}$, after castration as described in previous research.[16] Then, cells were cultured in WIT-P medium. Los Angeles prostate cancer-4 (LAPC-4) cells line, an androgen-dependent human PC cells, were cultured in RPMI 1640 containing 10% FBS (v/v) and 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were incubated in a 37 ℃ humidified incubator with 5% CO_2 and passed at 80%–90% confluency.

Cell Proliferation Assay

Cell proliferation assay was accessed with a Cell Titer 96 AQ_{ueous} one solution cell proliferation assay kit. Briefly, $PTEN^{-/-}/p53^{-/-}$ PC and LAPC-4 cells were seeded in 96-well plates at a density of 4,000 cells per 50 μL medium per well, and incubated at 37 ℃,

5% CO_2 for 24 h to allow for attachment. The cells were treated in triplicates with 8 nmol/L–2 μmol/L GA for either 24 or 48 h. Then 20 μL of the assay reagents was added and incubated for 1.5 h followed by shaking until all the crystals were dissolved. Subsequently, the optical absorbance at 490 nm (*A*490) was recorded. The experiment control cells were treated with 0.01% DMSO. Cell viability (%)=$[(1-A_{treatment})/A_{control}] \times 100\%$.

3D Cell Culture and Sphere Forming Assay

In order to get spheres, $PTEN^{-/-}/p53^{-/-}$ PC cells were passed through 21-, 22-, 23-, 25-, 27-, 30-gauge needles for 5 times, and a concentration of 500 single cell/well was suspended in organoid culture conditions, consisting of embedding cells within a Matrigel™ matrix and incubating with WIT-P media as described previously.[17] The mixture was then plated gently around bottom rim of each well in a 6-well plate, after incubating at 37 ℃ for 1 h for solidify, 1 mL medium was added into the center of the each well. After incubating for 24 h, cells were divided into 3 groups, treated with 50, 100 and 150 nmol/L GA, respectively. The control was treated with 0.01% DMSO. Media were changed every two days for 1 week. Organoids were then visualized and counted under the microscopy (10×).

Cell Viability Assay of Organoids

For the cell viability assay of PDX170.2 organoids, which were originally from a castrate resistant prostate cancer patient biopsy, cells were seeded at a density of 15,000 cells each well on a 48-well plate. After 24 h, cells were divided into 4 groups, treated with 0.5, 1, 1.5 and 2 μmol/L GA, respectively. The control was treated with 0.01% DMSO. Media were changed every two days for 2 weeks. CTG assay was performed to determine the cell viability in organoids according to the manufacturer's instructions. Briefly, after 150 μL DMEM was added into each well with 150 μL assay reagent, mixed the contents vigorously for 5 min to lyse cells. After incubation at room temperature for an additional 25 min, 100 μL of the cell lysis was transferred into a 96-well plate and the luminescent signal was read under Infinite 200 PRO multimode reader (Tecan, Männedorf, Switzerland).

Flow Cytometry

Analyses of cell cycle regulation were performed with propidium iodide (PI) by flow cytometry. Cells treated with 500 nmol/L GA for 6 and 8 h were trypsinized, then fixed in 70% ethanol and incubated at 4 ℃ overnight. Fixed cells were then re-suspended in PBS containing 2% 1 mg/mL PI (v/v), 0.2 mg/mL RNase A and 1% triton X-100 (v/v). After incubated for 30 min in dark, cell cycle distribution was analyzed in the Calibur (BD Biosciences, Heidelberg, Germany). DMSO (0.01%) was set as control. Cell cycle distribution was analyzed using Mod Fit LT.

Western Blot

Cells cultured in 2D and 3D were treated with 500 nmol/L GA for 4, 6, 8 h and 12, 24, 48 h, respectively. The control was treated with 0.01% DMSO. Total protein was harvested with RIPA buffer. Equal volume of total protein was loaded. After transferring, PVDF membrane was blocked for 1 h at room temperature. After that, the membrane was incubated with specific primary antibodies at 1:1000 dilution in PBST at 4 ℃ overnight, washed thrice with PBST, incubated with specific secondary antibodies at 1:2000 dilution in PBST for 1 h at room temperature and detected.

Reverse Transcription Polymerase Chain Reaction

For real-time reverse transcription polymerase chain reaction (RT-PCR) analysis, total ribonucleic acid (RNA) was isolated using a RNeasy mini kit. Reverse transcription of cDNA and PCR was performed as described, previously.[18] All reactions were normalized to mouse GAPDH and run in triplicate using primers as below: c-fos: Forward (5'-TCTGAGGAGGCCTTCACCCT-3')/Reverse (5'-TCTGAGGAGGCCTTCACCCT-3'), GAPDH: Forward(5'-AACGTCAAGAAGCAGTGTGC-3')/Reverse (5'- TCCAGCTCTGTCAGCTTGTT-3').

Statistical Analyses

All data were expressed as mean ± standard error (SE) of three independent experiment and analyzed with Graph Pad Prism 7.0 software. Differences among individual groups were determined by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's post test for comparisons among 3 or more groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

GA Inhibited Cell Proliferation in Mouse and Human PC Cells

As shown in Figure 2A, GA inhibited cancer cells'

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proliferation in a concentration- and time-dependent manner with an half maximal inhibitory concentration (IC_{50}) of 185.4 and 105.3 nmol/L as for 24 and 48 h, respectively. Meanwhile, as shown in Figure 2B, LAPC-4 cells were remarkably inhibited by GA with an IC_{50} of 312.5 and 154.3 nmol/Las for 24 and 48 h. Strikingly, 2 μmol/L GA led to a complete cell death in all cancer cells.

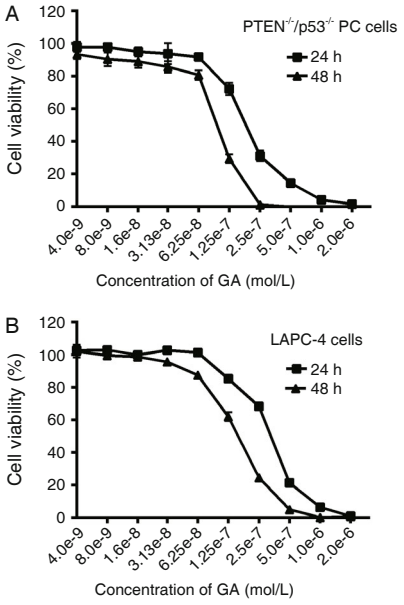

Figure 2. GA Inhibited Cell Proliferation in Mouse and Human PC Cells

Notes: (A) $PTEN^{-/-}/p53^{-/-}$ PC cells and (B) human PC LAPC-4 cells were incubate with 8 nmol/L-2 μmol/L GA for 24 and 48 h, results were presented by cell viability relative to control group.

GA Inhibited Organoids Growth of $PTEN^{-/-}/p53^{-/-}$ PC Cells

When $PTEN^{-/-}/p53^{-/-}$ PC cells were grown in 3D with matrix support, a small number of survival single cells generated organoids (Figure 3A), which indicated the existence of cancer stem cells (CSC).[19] Meanwhile, relative organoids numbers remarkably and progressively decreased from 30.5%±1.8% (control) to 21.1%±1.1%, 11.3%±1.4% and 2.4%±1.6%, corresponding to 50, 100, 150 nmol/L GA (Figure 3B).

GA Inhibited PDX170.2 Organoids Growth *Ex Vivo*

As shown in Figure 4, GA concentration-dependently inhibited PDX170.2 organoids numbers. And, cell viability progressively decreased to 86.5%, 55.4%, 29.2% and 8.1% with 0.5, 1, 1.5 and 2 μmol/L GA, respectively (Figure 4B).

GA Induced Mitochondrial Apoptotic Cell Death of $PTEN^{-/-}/p53^{-/-}$ PC Cells in 2D and 3D

As shown in Figure 5A, 500 nmol/L GA resulted in a noticeably increased Sub G_1 peak from 1.98% (0 h) to 11.78% (6 h) and 29.94% (8 h). Accordingly, visualized mitochondrial morphological change with confocal microscopy revealed that mitochondria were fragmented from a filamentous tubular network into punctuate and spherical organelles under GA treatment for 4 h as shown in Figure 5B.

Furthermore, as shown in Figure 5C, GA induced activation of caspase-3 and -9 as well as a conversion of LC3-Ⅰ to Ⅱ in both 2D and 3D culture as early as 6 and 48 h.

Involvement of Down-regulated MAPK Pathway in GA Induced Apoptosis

As shown in Figure 6A, protein levels of

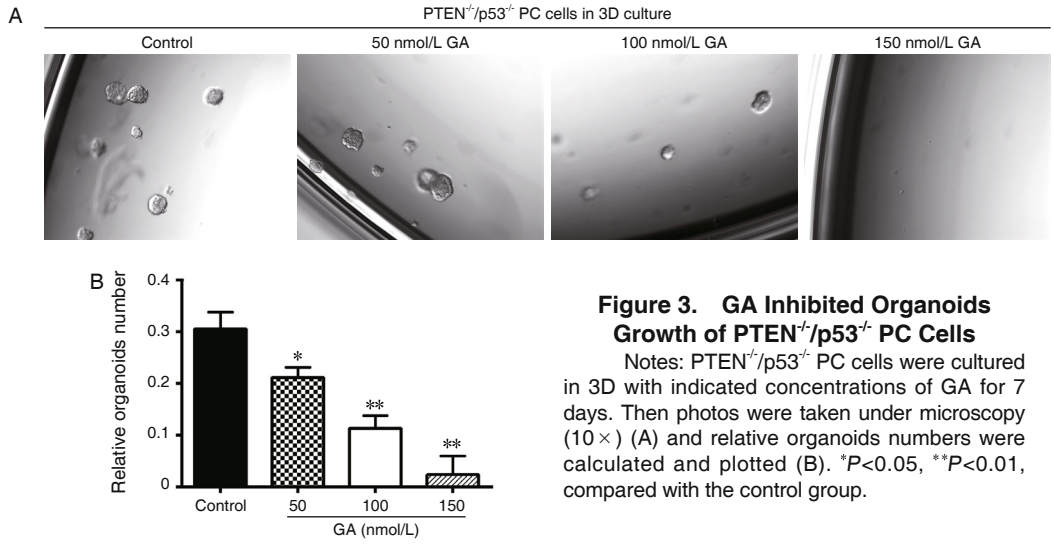

Figure 3. GA Inhibited Organoids Growth of $PTEN^{-/-}/p53^{-/-}$ PC Cells

Notes: $PTEN^{-/-}/p53^{-/-}$ PC cells were cultured in 3D with indicated concentrations of GA for 7 days. Then photos were taken under microscopy (10×) (A) and relative organoids numbers were calculated and plotted (B). $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group.

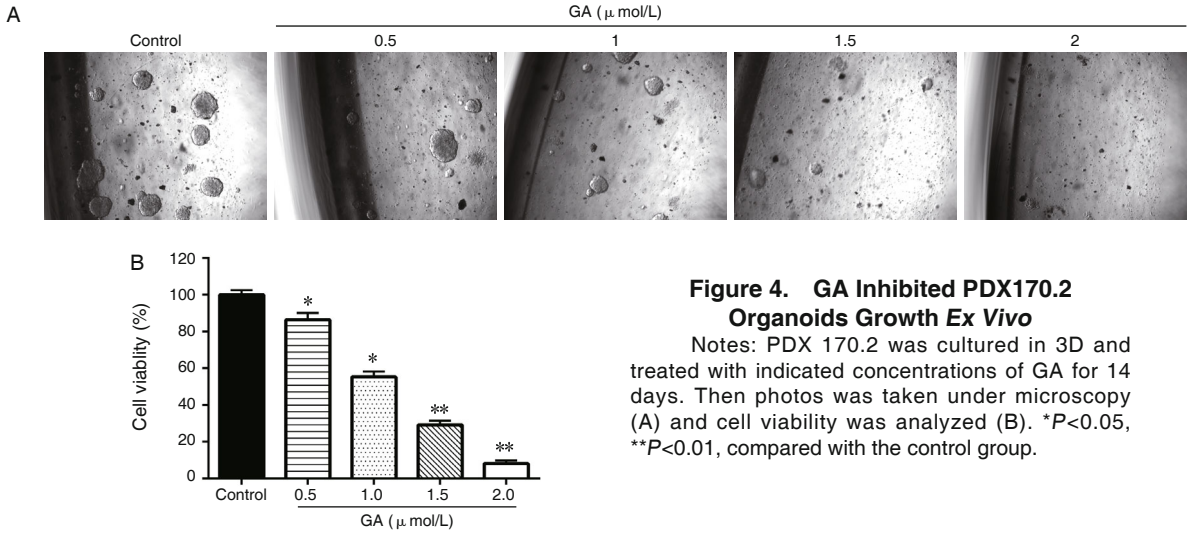

Figure 4. GA Inhibited PDX170.2 Organoids Growth *Ex Vivo*

Notes: PDX 170.2 was cultured in 3D and treated with indicated concentrations of GA for 14 days. Then photos was taken under microscopy (A) and cell viability was analyzed (B). *P<0.05, **P<0.01, compared with the control group.

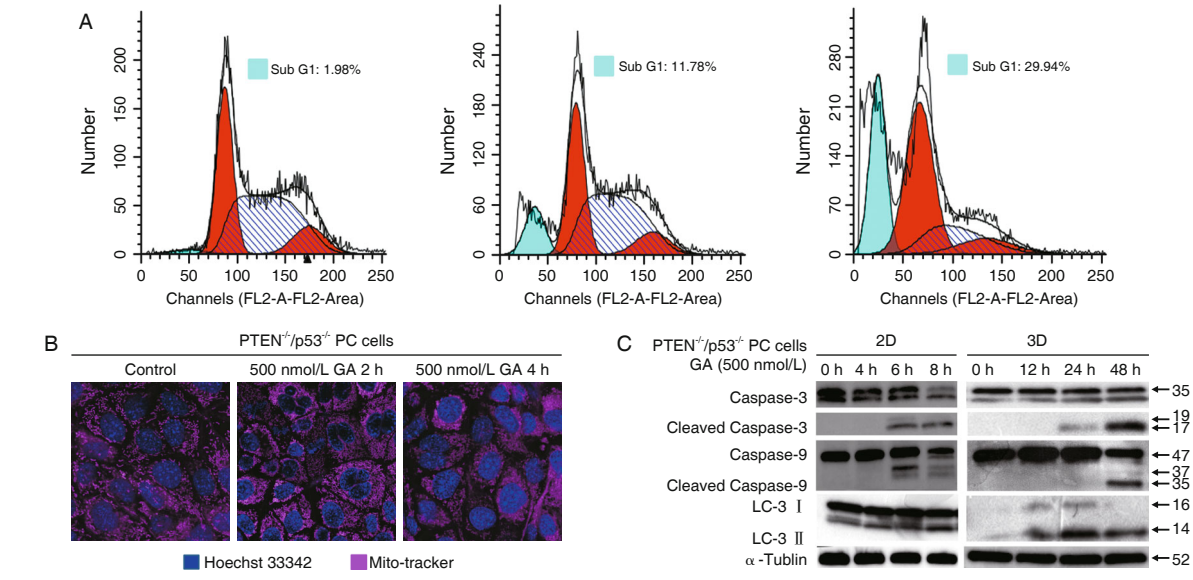

Figure 5. GA Induced Cell Apoptosis and Autophagy in 2D and 3D

Notes: (A) Flow cytometry of cells stained with PI under GA. (B) After incubated with GA stained cells were visualized under microscopy (20×). (C) Proteins expression levels of GA treated cells comparing to control.

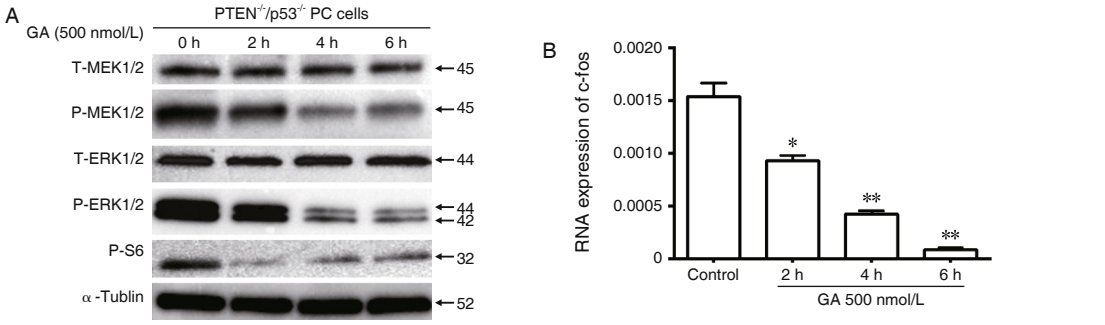

Figure 6. GA Down-Regulated MAPK Pathway

Notes: Protein expression levels of key nodes of MAPK pathway (A) and RNA expression level of c-fos (B) were analyzed in GA treated cells by WB and qRT-PCR, respectively. *P<0.05, **P<0.01, compared with the control group.

P-MEK1/2, P-ERK1/2 and the downstream P-S6 all started to decrease after 500 nmol/L GA as early as 2 h treatment. In addition, qRT-PCR (Figure 6B) revealed that expression level of c-fos, a proto-oncogene, was strongly inhibited under GA treatment for 2 h.

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DISCUSSION

Emerging evidences have suggested the natural products as important sources to develop novel chemotherapeutic agents.[20] GA is a small molecule that is present in the Chinese medicine gamboges,[21] which is undergoing a phase Ⅱ clinical trials in solid cancer therapy approved by the Chinese Food and Drug Administration.[22] GA has been extensively studied for its anti-tumor effect including anti-proliferative activities, DNA damage and apoptosis induction via the mitochondrial and some other pathways.[23-25] Thus, our present study showed that GA resulted in a significant growth inhibition in both mouse and human PC cells.

Despite the prevalence of PC, research has been hampered by several well-documented technical limitations, including the lack of *in vitro* model systems due to the limited number of PC cell lines available, which do not represent the diverse phenotypes of clinical disease. Nevertheless, recently described prostate organoid system which allows 3D organization of both benign and malignant PC cells extracted directly from patients.[26] Previous researches have established a mutual PC organoids model as for the research on the castration resistant PC from $PTEN^{-/-}/p53^{-/-}$ genetic engineered mice.[19] With the advantage of the organoids culture of $PTEN^{-/-}/p53^{-/-}$ PC cells, our research validated GA's anti-tumor effect in the most aggressive genetical mutated PC.

PDX models of PC have been generated previously by engraftment of human PC cells into immune-compromised and harvested after one generation or after serially transplantable grafts for multiple generations, both of which closely resemble the tissue architecture and heterogeneity of patient tumors.[27,28] After years of research, the PDX model have been proven to be a better *ex vivo* model for translational and precision medicine, which showed better prediction. Through, we haven't done *in vivo* experiment to validate GA's effect against aggressive PC, PDXs model was also an effective model. In our experiment, GA strongly and concentration-dependently inhibited the growth of PDX170.2 by CTG assay. In summary, our experiment used the conventional and advanced culture models represented the clinically castrate resistant PC and proved GA as a potent and promising anti-tumor compound for PC. It is indicated that future clinical utility of GA in castrate resistant PC which remain a challenge of PC treatment would be a new therapeutic strategy.[29]

Apoptosis is a form of programmed cell death that typically leads to activation of caspases via two major routes: the extrinsic death receptor and the intrinsic mitochondrial pathways.[30] Previous experiments have demonstrated GA as a strong apoptosis inducer.[11-13] In a study on RAJI cells, GA resulted in the activation of procaspase-3 and downregulation of antiapoptotic B cell lymphoma-extra large (Bcl-xL) gene expression.[31] Furthermore, the dose-dependent effect of GA on human gastric cancer cells (SGC-7901) and malignant melanoma cells (A375) was observed via upregulation and downregulation of pro-apoptotic and antiapoptotic gene expression.[32] In addition, several studies reported GA-mediated apoptotic cell death through the elevation of reactive oxygen species level in SMMC-7721 and MDA-MB 231 cell lines.[33,34] Interestingly, our study demonstrated that GA most likely induced mitochondrial cell apoptosis in aggressive PC cells in both 2D and 3D culture. In respect of that, mitochondrial apoptotic pathway related proteins: caspase-3 and -9 were detected after the addition of GA for 6 h. Furthermore, GA treatment led to mitochondrial fragmentations as early as 4 h, which is a typical morphological mitochondrial change of cell undergoing apoptosis that have been firstly demonstrated by Frank, et al[35] and confirmed by others.[36] Taken together, the results enclosed GA as an intrinsic apoptotic cell death inducer in aggressive form of PC.

To elucidate the mechanism of GA induced apoptosis, we investigated the role of mitogen-activated and stress-activated protein kinases (MAPKs), as ERKs have been demonstrated in PTEN or p53 mutated cells. ERK1/2, one of the most prominent members of the MAPK family, are phosphorylated and activated by specific MEKs.[37] c-fos, one of the first studied proto-oncogens, has been shown to be overexpressed and a critical inducers of apoptotic cell death among PC especially castration resistant ones.[38,39] It has been reported that activation of ERK1/2 pathway is sufficient for c-fos induced cell death in PC.[40,41] We observed that a treatment of $PTEN^{-/-}/p53^{-/-}$ PC cells with 500 nmol/L GA robustly and progressively suppressed the expression of P-MEK1/2, P-ERK1/2 protein levels and c-fos RNA level as early as 2 h, suggesting that

GA specifically inhibited the MAPK pathway and c-fos expression to induced cell apoptosis in castrate resistant PC.

In summary, the present results provided new evidences of understanding the effect of GA regulated cell apoptosis and set a solid role of GA in the castrate resistant PC. Meanwhile, we revealed GA as a possible c-fos inhibitor via suppressing the MAPK pathway and leading to cell apoptosis after all. In respect to the limited efficient therapy to PC, especially the castrate resistant phenotype, GA was proved as a promising anti-tumor drug against PC especially the c-fos over-expressed phenotypes.

Conflict of Interest

The authors declared no conflict of interest.

Author Contributions

Pan H and Lin HS conceived and designed the experiment. Pan H and Lu LY performed the experiment, Pan H and Wang XQ analyzed the data. Pan H and Kelly K wrote the manuscript and Lin HS did the prove reading.

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(Received December 22, 2016; First Online June 3, 2017)
Edited by ZHANG Wen