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## Original Article

# Gallic acid inhibits bladder cancer cell proliferation and migration via regulating fatty acid synthase (FAS)



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## ARTICLE INFO

## Article history:

Received 23 April 2017

Received in revised form

12 June 2017

Accepted 14 June 2017

Available online 8 July 2017

## Keywords:

Gallic acid

Bladder cancer

Fatty acid synthase (FAS)

Proliferation

Migration

## ABSTRACT

Bladder cancer is known as the world's ninth most prevalent cancer in 2012. New cytotoxic drugs have created considerable progress in the treatment. Gallic acid (GA) has been shown to inhibit carcinogenesis in animal models and various cancer cell lines. The aim of the present study was to evaluate the effect of GA on proliferation and migration inhibition of a bladder cancer cell line. The results showed that GA inhibited fatty acid synthase (FAS) activity and increased ER alpha level of TSGH-8301 bladder cancer cell. GA regulated the cell proliferation via the PI3K/AKT and MAPK/ERK pathway. Immunoprecipitation assay demonstrated that GA decreased Skp2 protein level and attenuated Skp2-p27 association. It was suggested that GA acted upstream of the proteasome to control p27 levels and ultimately inhibited G2/M phase transition. Further, transwell chambers assay showed that GA suppressed bladder cancer cell invasion and migration through p-AKT/MMP-2 signaling pathway. The finding indicated that GA inhibited TSGH-8301 bladder cancer cell growth, invasion and migration through inhibition of fatty acid synthase.

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## 1. Introduction

Bladder cancer is the ninth most common cancer in the world, and the incidence of bladder cancer is nearly 3 times higher in

more developed countries compared to less developed countries [1]. Many risk factors have been involved in tumorigenesis of bladder such as virus infection and smoking [2]. However, recent studies inferred that obesity increases the

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<http://dx.doi.org/10.1016/j.jfda.2017.06.006>

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risk of bladder cancer by approximately 10% [3]. Fatty-acid synthase (FAS) is a key biosynthetic enzyme involved in lipogenesis with the ability to catalyze a reductive *de novo* synthesis of long-chain fatty acids from acetyl coenzyme A (CoA) and malonyl-CoA. It plays an important role in energy homeostasis by converting excess carbon intake into fatty acids for storage [4]. Except the adipose and liver tissues, FAS expression in most normal tissue types is low. However, FAS is overexpressed in many cancers and has been strongly linked to tumor cell proliferation and apoptosis [5–7]. A previous study has reported FAS overexpression in bladder transitional cell carcinoma, and inhibition of FAS suppressed phosphorylated AKT (p-AKT) and induced apoptosis in bladder cancer [8].

Although exact mechanism about FAS overexpression in tumors is still unclear, the identification of a novel FAS/estrogen receptor alpha (ER alpha) fusion transcript expressed in a variety of human cancer cell lines suggests a close linkage between FAS and the ER alpha signaling pathway [9]. Expression of ER alpha and ER beta in bladder cancer was reported in some of the androgen receptor studies [10–12]. The studies supporting a role for ER alpha contrasts with that for ER beta in bladder cancer. For instance, ER beta could play positive roles in promoting bladder cancer cells progression via MCM5 regulation [13]. ER alpha knockout mouse models supported an ER alpha protective role in cancer initiation and growth, through modulating the activity of INPP4B/AKT pathways [14].

Gallic acid (3, 4, 5-trihydroxybenzoic acid; GA) is a type of phenolic organic compound which is widely distributed in natural plants and fruits, such as gallnuts, sumac, grape, green tea, oak bark, strawberry, lemon, banana, pineapple, witch hazel, and apple peel [15]. GA and its analogs polyhydroxyphenolic compounds have been reported to have many biological activities, including antioxidant, anti-mutagenic, and anti-carcinogenic [16,17]. However, the main interest in gallic acid and its analogs is related to its antitumor activity. In fact, anti-cancer activity of GA has been reported in various cancer cells, including prostate, lung, gastric, colon, breast, cervical and esophageal cancer [18]. It has been shown that propyl, lauryl, methyl gallate inducing apoptosis and inhibits proliferation in tumor cell lines is associated with oxidative stress, but no cytotoxicity against endothelial cells and normal fibroblast [19,20]. Some studies have shown that GA causes DNA fragmentation and is also responsible for the suppression of tumor angiogenesis, leading to inhibition of tumor metastasis, indicating the multiple anti-tumorigenesis of GA [20].

Anti-tumorigenesis of GA by inhibiting cell proliferation and inducing apoptosis is well known [21–23]. Our previously study also found that GA modulated Chk2-mediated phosphorylation of Cdc25c, causing G2/M phase arrest in bladder cancer [24]. Based on the unclear mechanism of FAS regulating ER alpha in bladder cancer cells and multiple anti-cancer effects of GA, we aimed to assay the capacity of GA in regulating FAS and ER alpha expression. We present evidence herein that GA modulated FAS mediated ER alpha, ERK and AKT phosphorylation to inhibit bladder cancer cells proliferation. GA also decreased SKP2 protein expression, causing G2/M phase arrest in bladder cancer. On the other hand, migration and invasion of Bladder cancer cells were prevented by GA via restraining MMP2 activation.

## 2. Method

### 2.1. Reagents

Gallic acid, 2-Propanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-butanol, dimethyl sulfoxide (DMSO), deoxycholic acid, dithiothreitol, EDTA, glycerol, Igepal CA-630, phenylmethylsulfonyl fluoride (PMSF), sodium chloride (NaCl), potassium chloride (KCl), sodium dodecyl sulfate (SDS), sodium phosphate, Tris-HCl and trypsin/EDTA used in the present study were purchased from Sigma Aldrich Chemical Company (St. Louis, MO). The reagents for electrophoresis were obtained from Bio-Rad Laboratories. Antibodies against AKT, phosphor-AKT, CDK1, cyclon B1, ER alpha, ERK2, phosphor-ERK, FAS, p21, p27, SERBP1, and SKP2 were from Santa-Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK).

### 2.2. Cell line and cell culture

Human urinary bladder cancer cells (TSGH-8301) were derived from a well-differentiated human TCC of the urinary bladder (Grade II, Stage A) and purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was changed twice a week, and the cells were subcultured when confluence was achieved.

### 2.3. Cell proliferation assay

Cell proliferation inhibition by GA was determined by thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, TSGH-8301 cells were seeded at a density of  $5 \times 10^4$  cells/mL in a 24-well plate overnight. Then, the cells were treated with GA at different concentrations (0, 50, 100, and 150  $\mu$ M) for various periods of time (24, 48, and 72 h). Then, the medium was changed and incubated with MTT solution (5 mg/mL)/well for 4 h. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm. After aspirating the supernatant, 200  $\mu$ L of dimethyl sulfoxide was added to each well to solubilize the formazan crystals formed in viable cells. The optical density was spectrophotometrically measured at 563 nm using enzyme-linked immunosorbent assay plate reader.

### 2.4. Western blot analysis

The cells were collected, the medium was removed and rinsed with PBS at room temperature. Then 0.5 mL of cold RIPA buffers (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) with fresh protease inhibitor was added. The supernatants were collected through the centrifugation at 12,000 rpm for 10 min. Cell lysate (50  $\mu$ g) was mixed with an equal volume of electrophoresis sample buffer and then boiled for 10 min, followed by analysis using SDS-PAGE and transfer of protein was from

the gel to nitrocellulose membrane (Millipore, Bedford, MA) by using electroblotting apparatus. Then the proteins were added with the ECL Western blotting detection reagents (Amersham Biosciences, USA) and analyzed using the Fuji LAS-3000 imaging system (Japan).

## 2.5. Immunoprecipitation

Cell lysates (500 µg protein/sample) were adjusted to 1 mL with lysis buffer and pre-cleared with protein A plus agarose for 1 h, then incubated overnight with primary antibody against p27<sup>Kip1</sup> and Skp2 plus agarose beads, and immunocomplexes were collected and washed three times with lysis buffer. The eluates were analyzed by immunoblotting with primary antibody against cyclin B1 and Cdc25C.

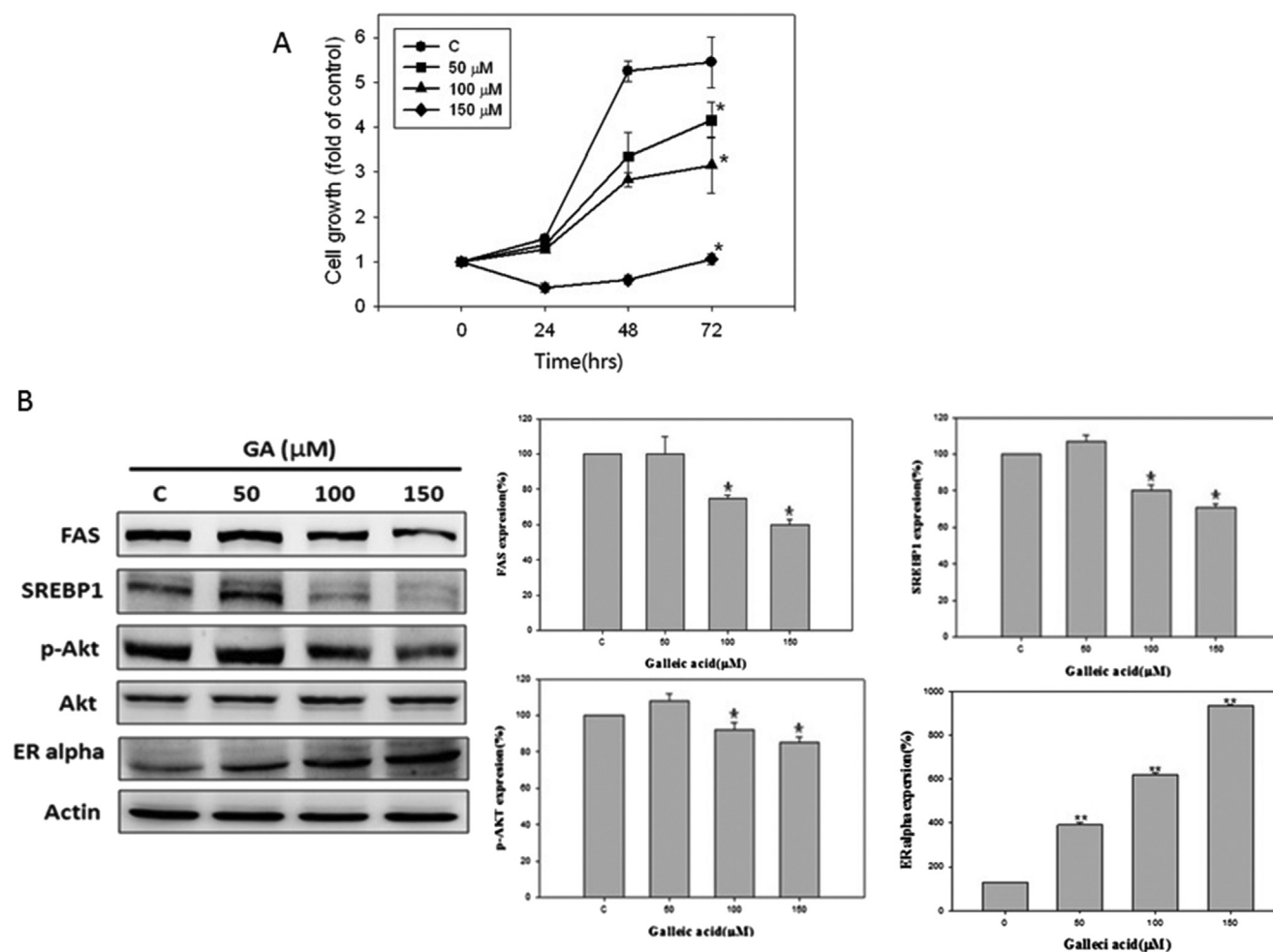
## 2.6. Migration and invasion assay

Cell migration and invasion assay was performed in Transwell chambers (24-well, 8-mM pore size, Corning). Before invasion

assay, the chamber was coated with metrigel previously. The serum-free DMEM (200 µL) containing  $1.0 \times 10^5$  cells and 1% bovine serum albumin were added into the top chamber of transwell with 200 µL of RPMI without FBS, whereas 800 µL of 20% FBS-contained RPMI-1640 was added in the bottom chamber as a chemoattractant. After the cell migration at 37 °C for 24 h, nonmigrating cells on the top of membrane were carefully removed by mechanical wiping. The cells that have migrated to the lower surface of membrane were fixed with 75% ethanol at 4 °C for 20 min and stained with 0.2% crystal violet for 15 min. After washing with PBS three times, the number of migrated cells in five random high-power fields ( $10 \times 10$ ) per membrane was counted an Olympus IX71 fluorescence microscopy.

## 2.7. Zymography assay

The activities of MMP2 and 9 were assayed by gelatin zymography. Firstly, samples were mixed with loading buffer and electrophoresed on 8% SDS–polyacrylamide gel



**Fig. 1** – GA inhibited TSGH-8301 cells proliferation and fatty acid synthesis. (A) Cells were treated with indicated concentration of GA (50–150 µM) for 0, 24, 48, 72 h. The cell viability in each well was quantified by using MTT assay. The results were represented as mean ± SD. \*,  $p < 0.005$  compared with control (C). (B) After treated with GA (50–150 µM) for 24 h, the protein levels of FAS, SREBP1, p-AKT, AKT, and ER alpha in TSGH-8301 cells as determined by Western blots. Actin protein was blotted as a control. The results were represented as mean ± SD. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  compared with control (C).

containing 0.1% gelatin at 140 V for 3 h. The gel was then washed twice in Zymography washing buffer (2.5% Triton X-100 in double distilled H<sub>2</sub>O) at room temperature to remove SDS. Following incubation at 37 °C in Zymography reaction buffer (40 mM Tris–HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, and 0.02% NaN<sub>3</sub>) overnight, the gel was stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol, and 10% acetic acid) for 1 h and destained with methanol/acetic acid/water (20:10:70, v/v/v).

### 2.8. Statistical analysis

Data were expressed as means ± SD of the three independent experiments. Statistical significance analysis was determined by using student's t-test comparisons with the control. The differences were considered significant for p values less than 0.05.

## 3. Result

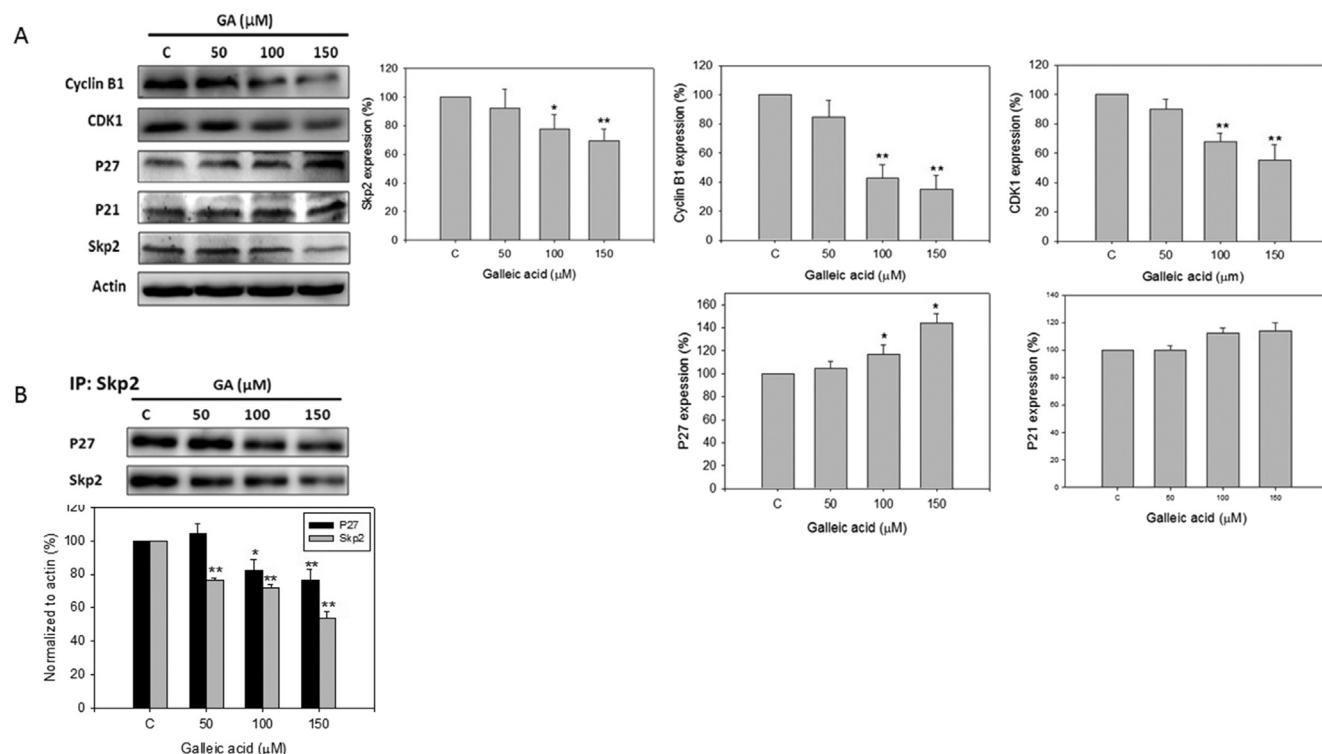
### 3.1. GA inhibited TSGH-8301 cells proliferation and fatty acid synthesis

Compared to the control group, MTT results showed the cell growth of GA groups was significantly lower, presenting dose and time relationship under various culture conditions (Fig. 1A). Excessive lipid biosynthesis is a characteristic feature of cancer. Deregulated fatty acid synthesis and

abnormal hormone expression promote the cancer cell survival [25]. Therefore, the enzymes in *de novo* fatty acid synthesis and related pathways were assayed. In Fig. 1B, GA decreased FAS and SREBP1 levels according to Western blotting method ( $p < 0.05$ ). AKT expression of TSGH-8301 cells between each group was not significantly different. However, GA inhibited phosphorylation of AKT activated by PI3K whereas ER alpha expression was induced by GA. Obviously, the results of MTT and Western blotting both suggested GA could inhibit cell proliferation and fatty acid synthesis.

### 3.2. GA inhibited TSGH-8301 cells proliferation via p27/Skp2 and ERK/ER signaling

In our previously study, GA could induce G2/M cell cycle arrest in TSGH-8301 cells via 14-3-3β related signaling [24]. The effect of GA on the G2/M transition regulate by Cyclin B1 and CDK1 were assayed here. After treatment with 100 or 150 M GA for 24 h, the expression of Cyclin B1 and CDK1 were decreased respectively (Fig. 2A). p21 and p27 are CDK inhibitors. GA increased p27 level in a dose-dependent manner whereas did not effect on p21 expression. Decreased Skp2 expression, a promoting aggressive tumor behavior of p27 degradation, was also observed after GA treatment. The protein lysate from control and GA-treated cells were immunoprecipitated using Skp2 antibody. In Fig. 2B, GA inhibited the binding of p27 with Skp2 significantly. These results indicated GA not only



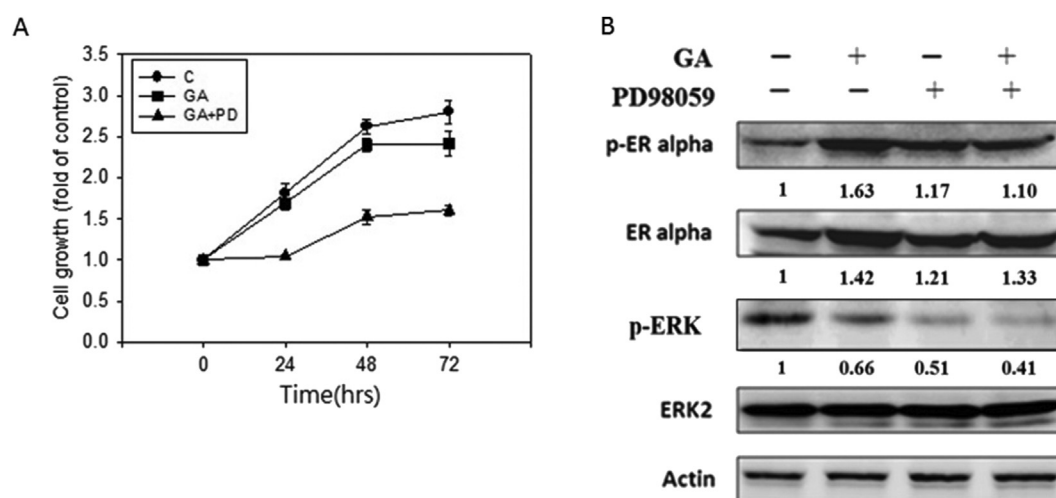
**Fig. 2 – Effect of GA on cell proliferation modulatory proteins in TSGH-8301 cells.** Cells were treated with various concentration of GA (50–150 μM) for 24 h. (A) The representative Western blots for the expression of Cyclin B1, CDK1, Skp2, P27 and P21 were assayed. (B) The immunoprecipitation for the expression of Skp2 and P27 were presented. Equal loading of protein was determined by β-actin antibody. The results were represented as mean ± SD. \*,  $p < 0.05$  and \*\*,  $p < 0.005$  compared with control (C).

inhibited Skp2 expression, but also decreased the interaction between Skp2 and p27 to increase p27 level.

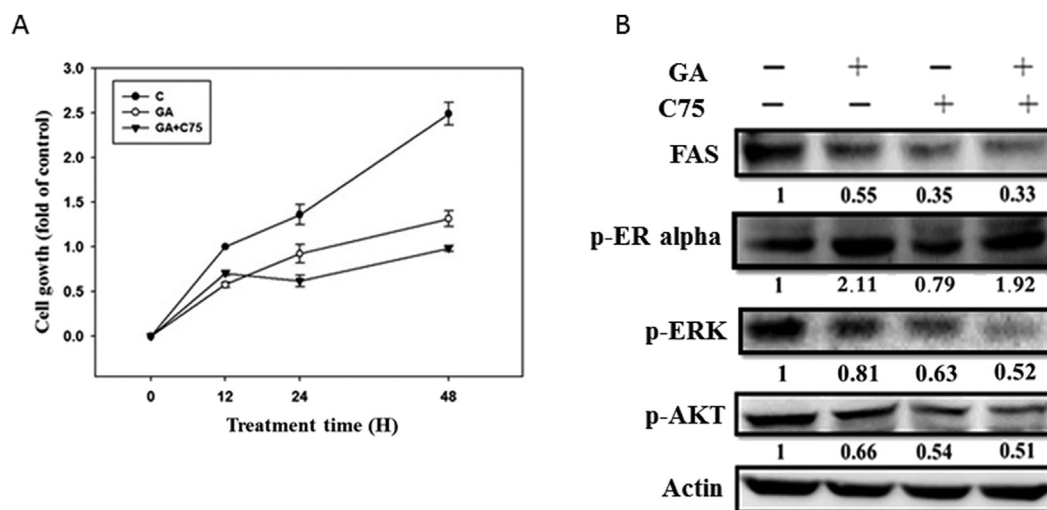
### 3.3. GA inducing ER alpha activation of TSGH-8301 cells was not through inhibiting ERK and FAS expression

Low to undetectable ER alpha expression is found in almost all human bladder cancer tissues. In Fig. 1B, GA inhibited phosphorylation of AKT activated by PI3K whereas ER alpha expression was induced by GA. We further examined the relationship between ER alpha and AKT related signaling such as ERK pathway. Co-treatment TSGH-8301 cells with GA and ERK inhibitor (PD98059, shorten as PD), cell viability indeed decreased compared with GA-treated alone group (Fig. 3A). Fig. 3B showed 100  $\mu$ M GA enhanced ER alpha phosphorylation

and inhibited ERK activation of TSGH-8301 cells. However, neither PD treatment alone nor co-treatment with GA and PD could raise the ER alpha expression in TSGH-8301 cells. On the other hand, lower level of phosphorylated ERK expression in PD + GA treated cells than PD or GA treated alone cells indicated that ERK signaling was associated with GA regulating TSGH-8301 cell viability, whereas was independent of ER alpha expression. In a variety of human cancer cell lines, a novel expressed FAS/ER alpha fusion transcripts suggest a close linkage between FAS and ER alpha signaling pathway [9]. To confirm the role of GA involving FAS and ER alpha, FAS inhibitor C75 was used to treat TSGH-8301 cells. Similarly as Fig. 3A, co-treatment TSGH-8301 cells with GA and FAS inhibitor (C75), cell viability decreased compared with GA-treated alone group (Fig. 4A). As expected, C75 and



**Fig. 3** – GA inhibited TSGH-8301 cells proliferation via ERK/ and ER signaling. Cells were pretreated ERK inhibitor PD98059 (PD) for 1 h and cotreated with GA 100  $\mu$ M for 24 h. Protein extracts were prepared and subjected to western blot analysis using ER alpha, p-ER alpha, p-ERK and ERK-2 and actin antibodies. Actin protein was blotted as a control.



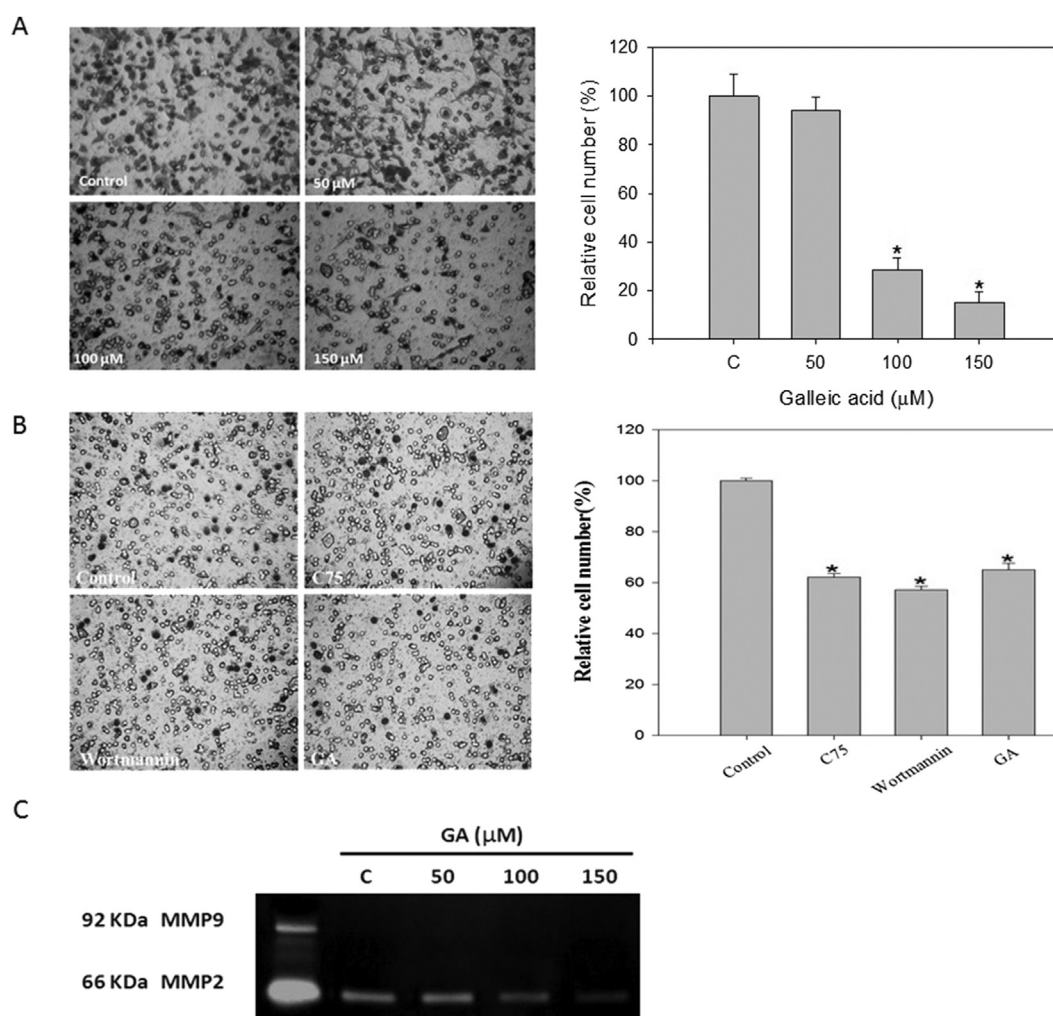
**Fig. 4** – GA induced ER $\alpha$  activation of TSGH-8301 cells by inhibiting ERK and FAS expression. Cells were pretreated C75 10  $\mu$ M for 1 h and cotreated with GA 100  $\mu$ M for 24 h. The most representative images of western blot for apoptosis related protein were assayed by Western blotting. Actin protein was blotted as a control (B).

100  $\mu$ M GA resulted in a 0.35 and 0.55-fold decrease in FAS level respectively (Fig. 4B). ERK and AKT activation were also inhibited by C75 and GA. Both GA alone and GA + C75 groups promoted ER phosphorylation but C75 reduced ER activation slight. This result implied that GA could reduce TSGH-8301 cells viability by inhibiting ERK and FAS expression via PI3K/AKT pathway. However, the mechanism of GA inducing ER activation was not related to ERK or FAS pathways.

### 3.4. GA inhibited the TSGH-8301 cells migration and invasion

Overexpression of PI3K/AKT and related proteins could promote the cancer cell migration and invasion [26,27]. Base on the observation that GA inhibited TSGH-8301 cells proliferation via PI3K/AKT pathway, we further investigated the role of GA in TSGH-8301 cells migration and invasion. 10% FBS was added in 24-well plate to induce cell migration and

the percentages of migrated cells decreased markedly in GA treated cells compared with the control group (Fig. 5A). The reduction of migration ability was 7%, 69%, and 81% at the concentration of 50, 100, and 150  $\mu$ M, respectively, compared to the control group. In Fig. 5B, the invasion ability of TSGH-8301 cells was inhibited by C75 and wortmannin. GA decreasing invasion of TSGH-8301 cells indicated that GA inhibited bladder cancer cell invasion by regulating FAS and PI3K signaling. Tumor migration and invasion require increased expression of MMP2/MMP9. To study whether the gelatinolytic activity of MMPs in TSGH-8301 cells could be inhibited by GA, zymographic analysis was performed. As shown in Fig. 5C, GA obviously reduced the gelatinolytic activity of MMP2 produced from TSGH-8301 cells. However, undetectable of MMP9 was observed in TSGH-8301 cells. These results revealed that GA inhibited the invasiveness of TSGH-8301 cells by decreasing the activity of MMP2.



**Fig. 5** – GA inhibited the TSGH-8301 cells migration and invasion. Migration and invasion analysis were done by Transwell chambers assay as detailed in Materials and Methods. Cells were treated with different concentration of GA (50–150  $\mu$ M) for 24 h. Transwells chambers assay was used to determine the cells migration (A) and invasion (B). Motility was quantified by counting the number of cells that migrated to the undersides of the membrane under microscopy (100 $\times$ ). The results were represented as mean  $\pm$  SD. \*,  $p < 0.005$  compared with control (C). (C) Replaced culture medium with serum-free RPMI for 24 h, the conditioned media was collected, and MMP-2 and MMP-9 activities were determined by gelatin zymography.

#### 4. Discussion

Owing to the rising incidence in developed countries and unestablished mechanisms of etiopathogenesis, to find successful and safe way of preventing bladder cancer progression is required urgently. The results of our study showed that GA in a dose- and time-dependent manner decreased cell proliferation and inhibited FAS mediated ER alpha, ERK and AKT phosphorylation of TSGH-8301 cells. By promoting SKP2 protein expression, GA caused G2/M phase arrest in bladder cancer. Furthermore, GA could inhibit the migration and invasion of Bladder cancer cells by restraining MMP2 activation. GA inhibitory effect on cell proliferation has been studied in several cancer cell lines in various studies [28–31]. It demonstrated the critical role on anti-cancer treatments and prevention.

Epidermal growth factor receptor (EGFR) activation and downstream signaling are closely related in cancer progression [32]. EGFR activates the adaptor or effector proteins and further stimulate their corresponding pathways, including PI3K/AKT and MAPK/ERK signaling, which leads to cell proliferation, survival, migration and angiogenesis [33]. MMP2 and MMP9 have been investigated in breast, rectal, ovarian, prostate, and bladder cancer, and its expression is reported to be increased primarily in high stage and advanced cancer. Over expression of MMPs may be associated with the recurrence of low grade bladder transitional cell carcinoma [34]. Hence, upregulation of several MMPs are associated with activation of the AKT [35]. In this study, GA made an effort to cancer prevention in initiation, progression, migration and invasion on bladder cancer.

Overexpression of FAS has been detected in multiple tumor types [36]. Many FAS inhibitors are in development and under preclinical evaluation. For instance, C75 suppresses the mitochondrial FAS pathway and impairs mitochondria function. Cerulenin attacks the FAS ketoacyl synthase (KS) domain, forming a covalent bond to the active site cysteine C1305. Orlistat is a novel inhibitor of the thioesterase domain of fatty acid synthase, an enzyme strongly linked to tumor progression. Unfortunately, none of these compounds have been tested in cancer patients due to limitations imparted by their pharmacologic properties or side-effect profiles. Over the last years, the number of searchers about polyphenols has increased gradually. In vitro and in vivo data demonstrated the potential of the polyphenols to treat and prevent cancer [37]. Therefore, the use of GA seems to contribute to anti-cancer therapy.

Men are three or four times more like to develop bladder cancer than women [38]. Except the lifestyle factors such as smoking, ER and AR expression are considered involved bladder cancer progression. ER alpha could inhibit the bladder cancer progression was demonstrated here and similar results were also proved by Miyamoto H. et al. [12]. Possible mechanisms such as via MAPK, AKT expression were established by several studies [14,39]. Recent researches reveal that estrogen protects against hepatic steatosis in female mice and down-regulation of hepatic TG synthesis [40,41]. Therefore, the relationship between estrogen and FAS in bladder cancer needs to be discussed. Further, to clarify the role of GA on regulating estrogen and FAS is our next task.

#### Conflicts of interest

The authors declare that they have no competing interests.

#### Acknowledgments

This investigation was supported by the Chung Shan Medical University and Cheng-Ching Hospital (CCGH-CSMU-104-001).

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