RESEARCH ARTICLE

Gallic acid induces G2/M phase cell cycle arrest *via* regulating 14-3-3β release from Cdc25C and Chk2 activation in human bladder transitional carcinoma cells

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Scope: Cell cycle regulation is a critical issue in cancer treatment. Previously, gallic acid (GA) has been reported to possess anticancer ability. Here, we have evaluated the molecular mechanism of GA on cell cycle modulation in a human bladder transitional carcinoma cell line (TSGH-8301 cell).

Methods and results: Using flow cytometer analysis, exposure of the cells to 40 μM GA resulted in a statistically significant increase in G2/M phase cells, which was accompanied by a decrease in G0/G1 phase cells. GA-treated cells resulted in significant growth inhibition in a dose-dependent manner accompanied by a decrease in cyclin-dependent kinases (Cdk1), Cyclin B1, and Cdc25C, but significant increases in p-cdc2 (Tyr-15) and Cip1/p21 by western blotting. Additional mechanistic studies showed that GA induces phosphorylation of Cdc25C at Ser-216. This mechanism leads to its translocation from the nucleus to the cytoplasm resulting in an increased binding with 14-3-3β. When treated with GA, phosphorylated Cdc25C can be activated by ataxia telangiectasia-mutated checkpoint kinase 2 (Chk2). This might be a DNA damage response as indicated by Ser-139 phosphorylation of histine H2A.X. Furthermore, treatment of the cells with a Chk2 inhibitor significantly attenuated GA-induced G2/M phase arrest.

Conclusion: These results indicate that GA can induce cell cycle arrest at G2/M phase *via* Chk2-mediated phosphorylation of Cdc25C in a bladder transitional carcinoma cell line.

Keywords:
Cell cycle / Checkpoint kinase 2 / G2/M phase / Gallic acid / Human bladder transitional carcinoma

1 Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid; GA) is a naturally occurring plant phenol obtained by the hydrolysis of tannins and is abundant phenolic compound in various natural products, including gallnuts, sumac, tea leaves, green tea, apple-peels, grapes, strawberries, and in red and white wine [1, 2]. It has a wide range of biological activities, including antioxidant, antimutagenic, and anticarcinogenic activity [3, 4], and is expected to reduce the risk of disease and brings health benefits through daily intake [5]. Other beneficial effects might be the antioxidative effects in senescence accelerated mice and could reinstate the activities of catalase and gluthation peroxidase in these animals [6]. GA possesses cytotoxicity against cancer cells and has antiinflamatory and antimutagenic properties [5]. It was described as a free

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Abbreviations: ATM, ataxia telangiectasia mutated; **CDK,** cyclin-dependent kinase; **CDKI,** CDK inhibitors; **Chk,** checkpoint kinase; **GA,** gallic acid; **PI,** propidium iodide; **TCC,** transitional cell carcinoma

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radical scavenger and an inducer of differentiation and programmed cell death in a number of tumors, including prostate cancer [7], leukemia [8], lung cancer [9], and melanoma [10]. Evidence is accumulating to indicate that GA can inhibit proliferation of cancer cells in culture by causing apoptosis and/or cell cycle arrest [11, 12]. Although considerable progress has been made toward our understanding of the mechanism of GA-induced apoptosis, the sequence of events leading to cell cycle arrest in GA-treated cells is poorly defined.

Bladder cancer is the fifth most common cancer in the United States, with an estimated 68 000 newly diagnosed cases and 14 000 deaths in the United States in 2008 [13]. Transitional cell carcinoma (TCC) is the most common bladder cancer (90%), which in ~80% of the cases occurs as superficial and in ~20% as invasive tumor [14]. Bladder cancers display mutations in cell cycle checkpoint genes that are associated with malignant growth and genetic instability [15]. Several studies have demonstrated an association between deregulation of cell cycle progression and development of cancer, suggesting that inhibition of unchecked cell cycle regulation in cancer cells could be a potential target for cancer treatment [16]. The regulation of cell cycle is controlled by a family of cyclin/cyclin-dependent kinase (CDK) complexes and the CDK inhibitors (CDKIs) [17]. G2/M transition is positively regulated by Cdc2 and Cyclin B complex [18] and the Cdc25 family of phosphatases regulate the activity of Cdc2 through dephosphorylation of inhibitory phosphorylation at threonine 14 and tyrosine 15, caused by Wee1 or Myt1 [19]. These phosphatases are inactivated through phosphorylation by cellular checkpoint kinases (Chk1/2), which may in turn be activated by upstream kinase ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad 3-related (ATR) in response to DNA damages [20].

In our previous study, we found that SLWLQY, a prescription in traditional Chinese medicine containing 16 herbs, possesses the ability to cause an irreversible arrest at G2/M phase and apoptosis in human bladder carcinoma cells and in BALB/c-nu mice [21]. The active compounds existing in the SLWLQY, including *Panax ginseng* C.A., *Astragalus membranaceus*, *Paeonia lactiflora* Pall, *Angelica sinensis* Diels, *etc.*, contain high levels of polyphenols, like GA, and it might be the bioactive constituent of SLWLQY in TCC cells [21]. Therefore, in the present study, we are aiming to assess the effect of GA in causing cell cycle arrest and the cell cycle regulating proteins, including Cyclin B1, Cdc2, and Cdc25C, in TCC cells. In addition, we are also examining the ATM/Chk2-mediated phosphorylation of Cdc25C at Ser-216, and the binding ability between phosphorylated Cdc25C and 14-3-3β. We hypothesize that GA modulates Chk2-mediated phosphorylation of Cdc25C, causing G2/M phase arrest in bladder cancer. Our studies may provide a rationale for future studies in evaluating prevention and intervention strategies of GA in bladder cancer pre-clinical models.

2 Materials and methods

2.1 Chemicals

Tris-HCL, EGTA, SDS, BSA, Nonidet P-40, DMSO, RNase A, Chk2 inhibitor, GA, and polyclonal antibody against α-actin were purchased from Sigma Chemical (St. Louis, MO, USA). Minimum essential medium, DMEM, RPMI medium 1640, F-12 nutrient mixture, fetal bovine serum, penicillin—streptomycin-mixed antibiotics, L-glutamine, Dulbecco's PBS, and trypsin-EDTA were purchased from Gibco/BRL (Gaithersburg, MD, USA). Nuclear and cytoplasmic fraction kit was obtained from CHEMICON (USA). Monoclonal antibody against Cdc2 and Cyclin B1 obtained from Upstate, Cip1/p21, Cdc25C, phospho-Cdc25C (Ser-216), Chk1, Chk2, phospho-Chk2 (Thr-68), 14-3-3β, and 14-3-3α from Santa Cruz Biotech, phosphor-ATM (Ser-1981), phosphor-cdc2 (Tyr-15), and phosphor-H2A.X (Ser-139) from Cell Signaling Technology Inc. (Beverly, MA, USA). The enhanced chemiluminescence kit was purchased from Amersham Life Science (Amersham, UK).

2.2 Cell line and cell culture

Human urinary bladder cancer cells (TSGH-8301) were purchased from the Bioresource Collection and Research Center (HsinChu, Taiwan). TSGH-8031 cells were derived from a well-differentiated human TCC of the urinary bladder [22], having wt p53 but mutant Rb gene. Cells were maintained in RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin, and 1% streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The media was changed every other day. Cells were seeded at a density of 6×10^5 onto each 10-mm Petri dish for 24 h before drug treatment. For the Chk2 inhibitor study, cells were treated with 20 μM Chk2 inhibitor for 2 h before GA treatment at 40 μM for 24 h.

2.3 Cell cycle analysis

Cell cycle analysis was performed with a flow cytometer (FACS Calibur; BD Bioscience, CA, USA). TSGH-8301 cells at 60% confluence were treated with either DMSO alone or 20–40 μM GA for 24 h. Then, the cells were washed twice with cold PBS solution. The cell suspension was then centrifuged at 1500 rpm for 5 min, fixed with 70% ethanol at −20°C overnight. Prior to the samples being analyzed by the flow cytometry, 1 mL of cold propidium iodide (PI) stain solution (20 μg/mL PI, 20 μg/mL RNase A, and 0.1% Triton X-100) was added to the mixture, and it was incubated for 15 min in darkness at room temperature. Data acquisition and analysis were performed in the flow cytometer with accompanying software (CellQuest, BD Bioscience, CA, USA).

2.4 Western blot analysis

The cells after treatment with DMSO (control) or the desired concentration of GA for the specified time interval were 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. Cells were scraped and the lysate was centrifuged at 10 000 × *g* for 10 min. Protein concentration in the supernatant was measured with Bradford method. Equal amounts (50 μg) of total protein from control and treated cell extracts were loaded onto 12% SDS-PAGE gels and transferred to PVDF membranes with Towbin buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol). Membranes were blocked with 5% nonfat milk in 0.05% Tween-20 in PBS for 1 h at room temperature and then incubated with primary antibody. The membranes were washed with 20 mM Tris, 500 mM NaCl, and 0.05% Tween-20 and incubated with secondary antibody (goat anti-mouse IgG at 1:10 000). Substrates were visualized by using echo-chemiluminescence (Amersham Biosciences, USA) and analyzed using the Fui 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. The mixture was then incubated overnight with primary antibody against 14-3-3β plus agarose beads, and immunocomplexes were collected and washed three times with lysis buffer. For immunoblotting, immunocomplexes or total cell lysates were denatured with 2 × sample buffer. Samples were subjected to SDS-PAGE on 12% gel and separated proteins were transferred onto membrane by western blotting. Membranes were blocked with blocking buffer for 1 h at room temperature and probed with primary antibody against Cdc25C overnight at 4°C followed by peroxidase-conjugated appropriate secondary antibody and enhanced chemiluminescence detection.

2.6 Preparation of nuclear and cytoplasmic fraction

Nuclear and cytoplasmic fractions from control (DMSO-treated) and GA-treated (40 μM for 12 h) TSGH-8301 cells were prepared by using nuclear and cytoplasmic fraction kit (CHEMICON). Briefly, cells were harvested by scraping and rinsed twice in ice-cold PBS. The cells were then swollen in cytoplasmic lysis buffer for 15 min. The cells were destroyed by passing through a syringe with a small gauge needle, and the nuclei were removed by centrifugation (8000 × *g*, 20 min). Both the supernatant (cytosolic fraction) and the nuclear extract were concentrated and used for western blot analysis. The blot was stripped and re-probed with α-tubulin or C23 antibody to ensure equal protein loading as well as to rule out cross-contamination of cytoplasmic and nuclear fractions.

2.7 Statistical analysis

Data reported are mean ± standard deviation of three independent experiments and are evaluated by one-way analysis of variance with *post hoc* Dunnett's test. A *p*-value of ≤ 0.05 was considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA).

3 Results

3.1 GA treatment induces irreversible G2/M phase arrest in TSGH-8301 cells

To investigate whether GA treatment imparts an anti-proliferative effect against bladder TCC cells, the effect of GA on cell cycle distribution is determined using a flow cytometer after staining of the cells with PI. The result shows that exposure of the cells to 30–40 μM GA results in a statistically significant increase in G2/M phase cells to about 21.5–30.6% compared with the control, which is accompanied by a decrease in G0/G1 phase cells (Fig. 1A). Based on this result, we selected the 40-μM dose and next assessed whether the growth-inhibitory effects of GA are accompanied by its effect on cell cycle progression following GA treatment for 6, 12, and 24 h (Fig. 1B). At time point of 24 h, compared with controls, GA treatment caused an arrest in G2/M phase (11.9% *versus* 31.6%, $p < 0.01$) (Fig. 1B).

3.2 Effect of GA on the expression of proteins that regulate G2/M transition

As GA treatment of cells caused cell cycle arrest at 24 h, we further observed the effects of GA treatment on the protein levels of Cdc2 and Cyclin B1 during G2 cell cycle progression. GA strongly decreased the progression of Cdc2 and Cyclin B1 levels in a dose-dependent manner (0.4-fold and 0.6-fold, Fig. 2A). A kinetics study starting at 6 h was carried out to examine the effect of 40 μM GA on the protein levels of cell cycle regulatory molecules. As shown in Fig. 2B, GA treatment of cells for varying time periods also resulted in a decreased in the protein levels of Cdc2 and Cyclin B1 at later time points of 12 and 24 h. We also observed an increase in phosphorylation of Cdc2 at Tyr 15 site, where the dephosphorylation form of Cdc2 is active and regulates the entry of cells into M phase [23]. The densitometric analyses of these blots showed maximum increase (1.7-fold) in the phosphorylation of Cdc2 as a functional time up to 12 h after GA treatment (Fig. 2B).

The other important regulator of CDKs is a family of inhibitory proteins known as CDKIs that bind to CDK–cyclin complex and negatively regulate CDK activity [24, 25]. Based on our observation of the cell cycle arrest effect of GA, we assessed whether this agent also modulated

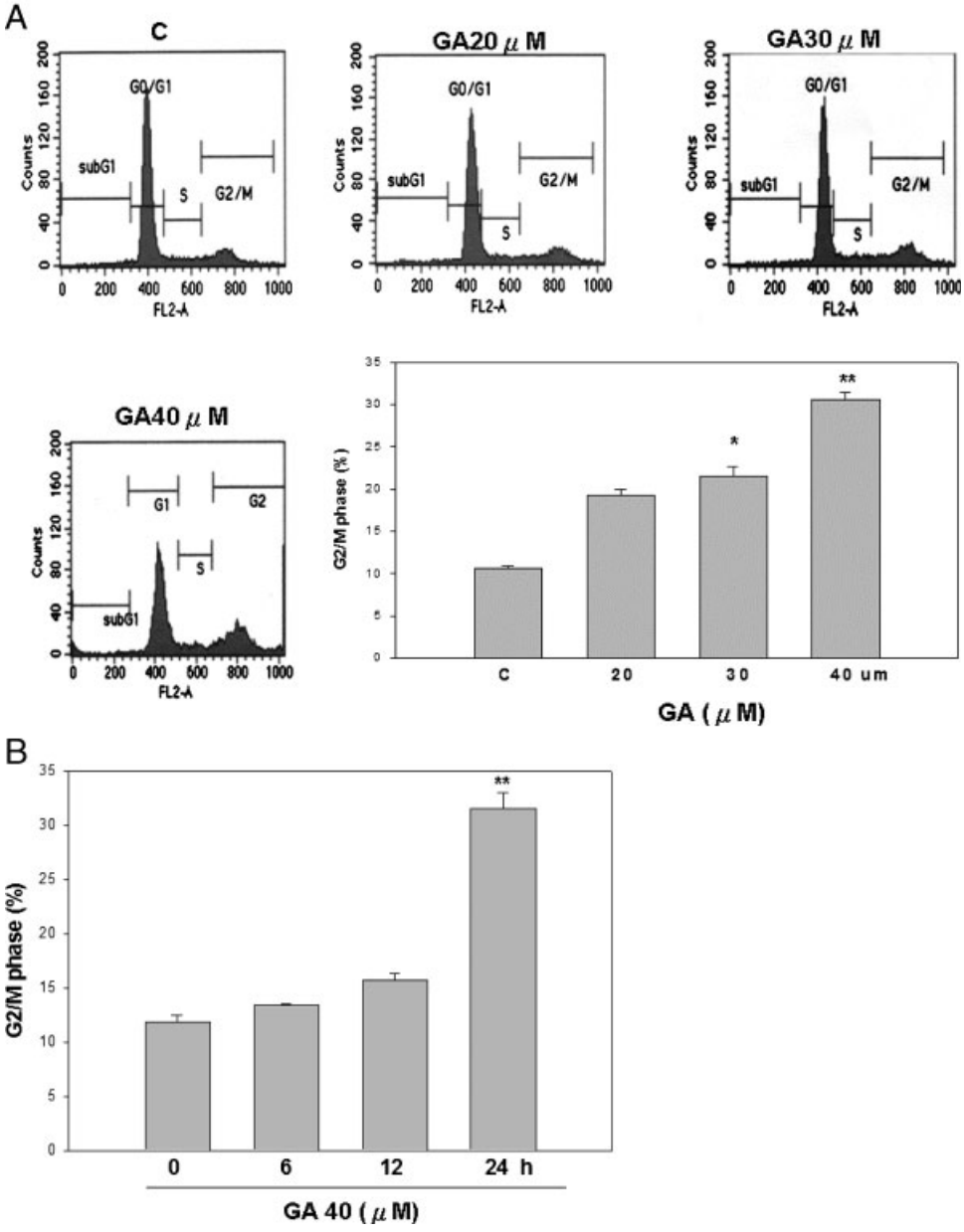

Figure 1. Effect of GA on cell cycle distribution in TSGH-8301 cells. The cells were treated with DMSO (control) or 20, 30, and 40 μM GA for 24 h (A), or 40 μM GA for 0, 6, 12, and 24 h (B). Subsequently, the cells were processed for analysis of cell cycle distribution by flow cytometry. The data shown in each case are mean ± SD of three independent samples and are representative of two independent experiments with comparable observations. $^{*}p<0.05$; $^{**}p<0.01$.

the levels of CDKIs. Our results reveal that the GA treatment (20, 30, and 40 μM) for 24 h induced the protein expression of Cip1/p21 in a dose-dependent manner (Fig. 3A). Cip1/p21 protein is seen expressed 3 h after GA treatment and persisted for 24 h (Fig. 3B). The densitometric analysis of the blots for Cip1/p21 shows maximum induction (1.9-fold) after 24 h of 40 μM GA treatments at without any noticeable change in Kip1/p27 levels (data not shown). Altogether, these results clearly shows that GA induces a decrease in the protein levels of Cdc2 and Cyclin B1 in TSGH-8301 cells and a selective induction in Cip1/p21; the regulating effect among these molecules might cause cell cycle arrest.

3.3 Effect of GA on Cdc25C (Ser-216) phosphorylation and total protein levels in TSGH-8301 cells

To gain insights into the mechanism of cell cycle arrest upon treatment with GA, the level of Cdc25C proteins is compared, and representative blots are shown in Fig. 3C. When compared with control, GA (40 μM) treatment of cells results in a decrease in total levels of Cdc25C (0.55-fold) at 24 h.

As Cdc25C plays critical roles in dephosphorylation of Cdc2 [23], we conducted a time point analysis of the molecules involved in G2/M phase of the cell cycle. Interestingly,

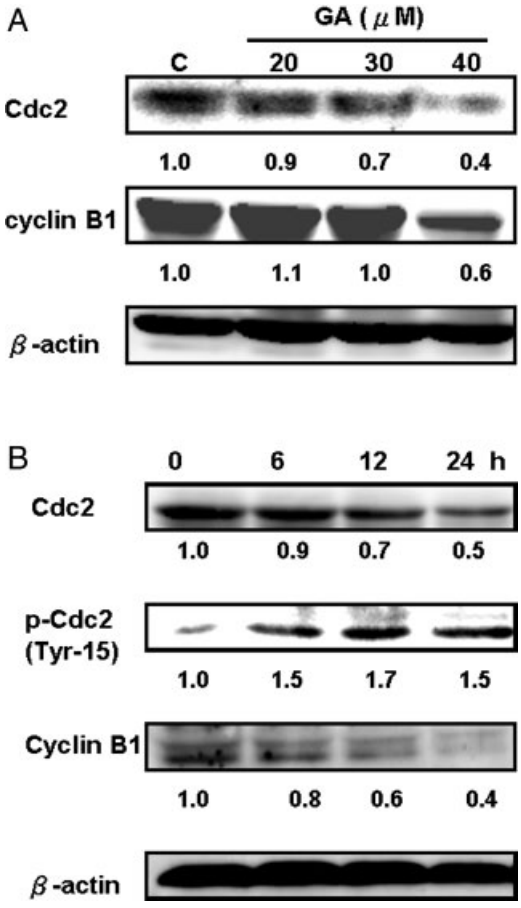

Figure 2. Effect of GA on the expression of Cdc2 and Cyclin B1 in the regulation of G2/M transition. Cells were cultured as described in the Section 2, and treated with either DMSO alone (control), or varying concentrations (20, 30, 40 μM of GA for 24 h (A), or 40 μM GA for 0, 6, 12, and 24 h (B). The data are representative of two independent experiments with comparable observations.

GA treatment of cells resulted in a strong phosphorylation of Cdc25C at Ser-216, which is evident as early as 12 h after GA (40 μM) treatment and persisted for the duration of the experiment (24 h post-treatment; Fig. 3C). Furthermore, GA treatment of cells resulted in a decreased in total levels of Cdc25C at 12 (0.8-fold) and 24 h (0.5-fold) (Fig. 3C).

3.4 GA promoted translocation of Cdc25C from the nucleus to the cytoplasm

Binding of Cdc25C with 14-3-3 prevents nuclear localization of this dual-specificity phosphatase. GA treatment of cells results in an increase in the expression of 14-3-3β protein levels, without change in 14-3-3α (Fig. 4A). Therefore, we are examining the effect of GA on the binding of Cdc25C with 14-3-3β. The protein lysate from control and

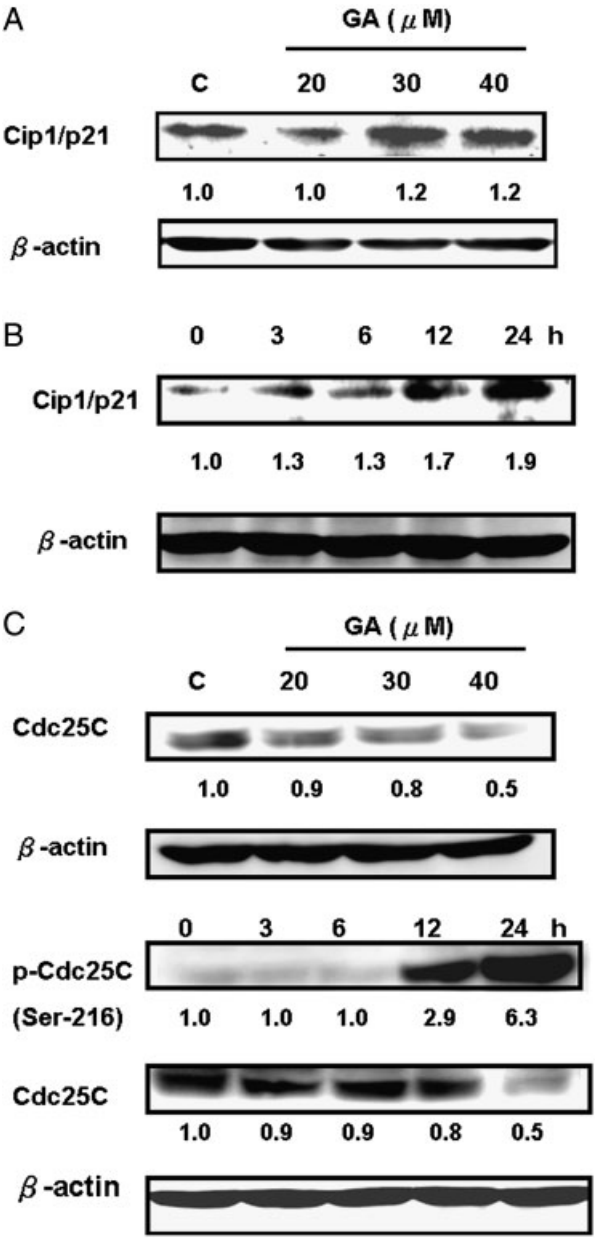

Figure 3. GA-induced Cip1/p21 protein expression and phosphorylation of Cdc25C in TSGH-8301 cells. Cells were cultured as described in the Section 2, and treated with either DMSO alone (control) or treated with varying concentration of GA for 24 h (A) or 40 μM GA for 0, 3, 6, 12, and 24 h (B). Immunoblotting for Cdc25C and phosphor-Cdc25C using lysates from control and GA-treated cells (C). Immunoblotting for each protein was performed two or more times using independently prepared lysates, and the results were comparable.

GA-treated cells (40 μM for 12 h) are immunoprecipitated using anti-14-3-3β antibody, and the immune complex is analyzed for the presence of Cdc25C by immunoblotting. As shown in Fig. 4B, GA increased the binding of Cdc25C with 14-3-3β as early as 12 h and a threefold increase by 24 h. These results suggest that GA treatment might lead to

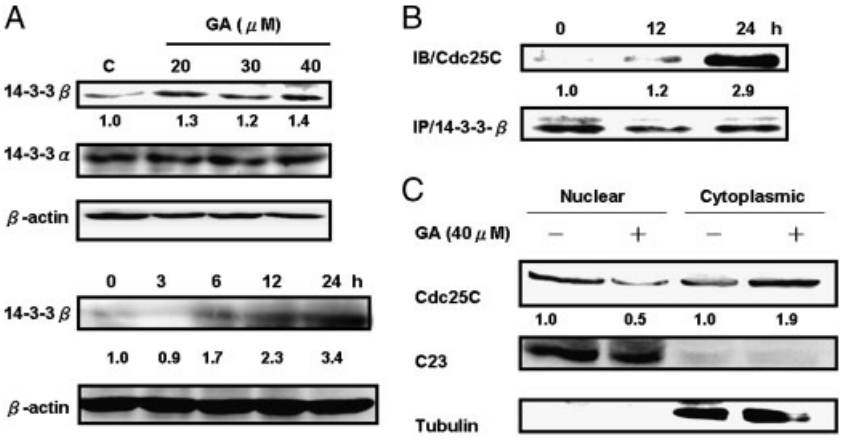

Figure 4. Effect of GA on binding of Cdc25C with 14-3-3β and on nuclear/cytoplasmic distribution of Cdc25C. Cells were cultured as described in Section 2 and treated with either DMSO (control) or varying concentration of GA for 24 h. (A) Immunoblotting for 14-3-3β and 14-3-3α using lysate from control and varying concentrations of GA-treated cells (A, upper panel); or cells treated with 40 μM GA for 0, 3, 6, 12, and 24 h (lower panel). (B) Effect of GA on binding of Cdc25C with 14-3-3β. Five hundred microgram of lysate proteins from control and GA-treated cells (40 μM for 12 or 24 h) were used for immunoprecipitation (IP) with 14-3-3β antibody followed by immunoblotting (IB) for Cdc25C. (C) Immunoblotting for Cdc25C using nuclear and cytoplasmic fractions prepared from control (DMSO) and GA-treated cells (40 μM for 12 h). Blots were probed with anti-C23 (middle panel) and anti-α-tubulin (bottom panel) antibodies to normalize for equal protein loading.

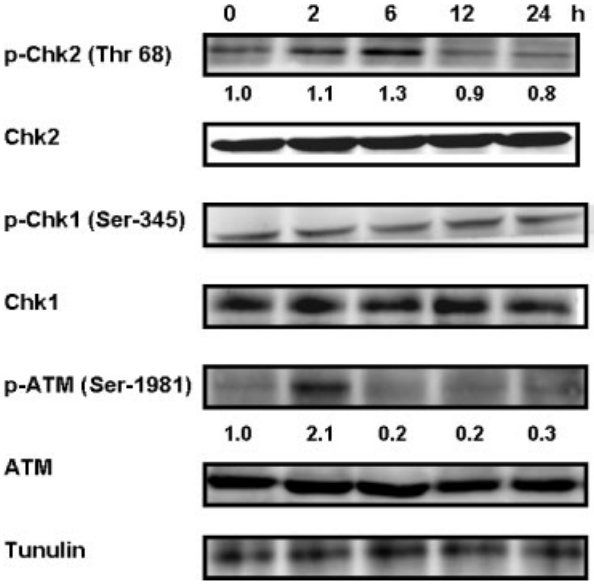

Figure 5. GA increases phosphorylation of Chk2 and ATM in TSGH-8301 cells. Cells were cultured as described in Section 2 and treated with either DMSO (control) or 40 μM GA for indicated time periods. Total cell lysates were prepared and analyzed for p-Chk2 (Thr-68), p-Chk1 (Ser345), p-ATM (Ser-1981), Chk1, and Chk2 protein levels by western immunoblotting.

translocation of Cdc25C from the nucleus to the cytoplasm because of increased binding with 14-3-3β.

Cytoplasmic accumulation of Cdc25C upon treatment with GA is further confirmed by biochemical fractionation of cytoplasmic and nuclear fractions, as shown in Fig. 4C. In DMSO-treated control, the intensity of Cdc25C immunoreactive band is significantly stronger in the lane corresponding to nuclear fraction than in the cytoplasmic fraction. Treatment of cells with GA results in a decrease in nuclear Cdc25C signal intensity, while there is a significant increase in cytoplasmic Cdc25C signal intensity (Fig. 4C). These results confirm that GA treatment indeed promoted translocation of Cdc25C from the nucleus to the cytoplasm.

3.5 GA treatment increased Ser-345 phosphorylation of Chk2

Several kinases, including Chk1 and Chk2, have been implicated in Ser-216 phosphorylation of Cdc25C [26, 27]. Chk1 and Chk2 are intermediaries of DNA damage checkpoints and activated by phosphorylation on Ser-345/Ser-317 and Thr-68, respectively [28, 29]. Therefore, we examined whether GA treatment affects phosphorylation of Chk1 or Chk2. Representative immunoblots for phospho-Chk2 showed increased Thr-68 phosphorylation of Chk2 over control, which was evident as early as 6 h after GA treatment and decreased by the end of experiment (Fig. 5). The level of total Chk2 protein was not affected by GA treatment. Additionally, GA treatment did not affect either the Chk1 protein level or its phosphorylation (Fig. 5).

In this regard, ATM and ATR are nuclear kinases recently identified as being activated in response to DNA damage/genotoxic stress in eukaryotic cells [30–32]. Based on our results, we hypothesized that GA can activate ATM/ATR kinases that are known to activate cell cycle Chk1/2 [31]. Immunoblotting using an antibody specific for phopho-ATM (Ser-1981) revealed increased phosphorylation of ATM

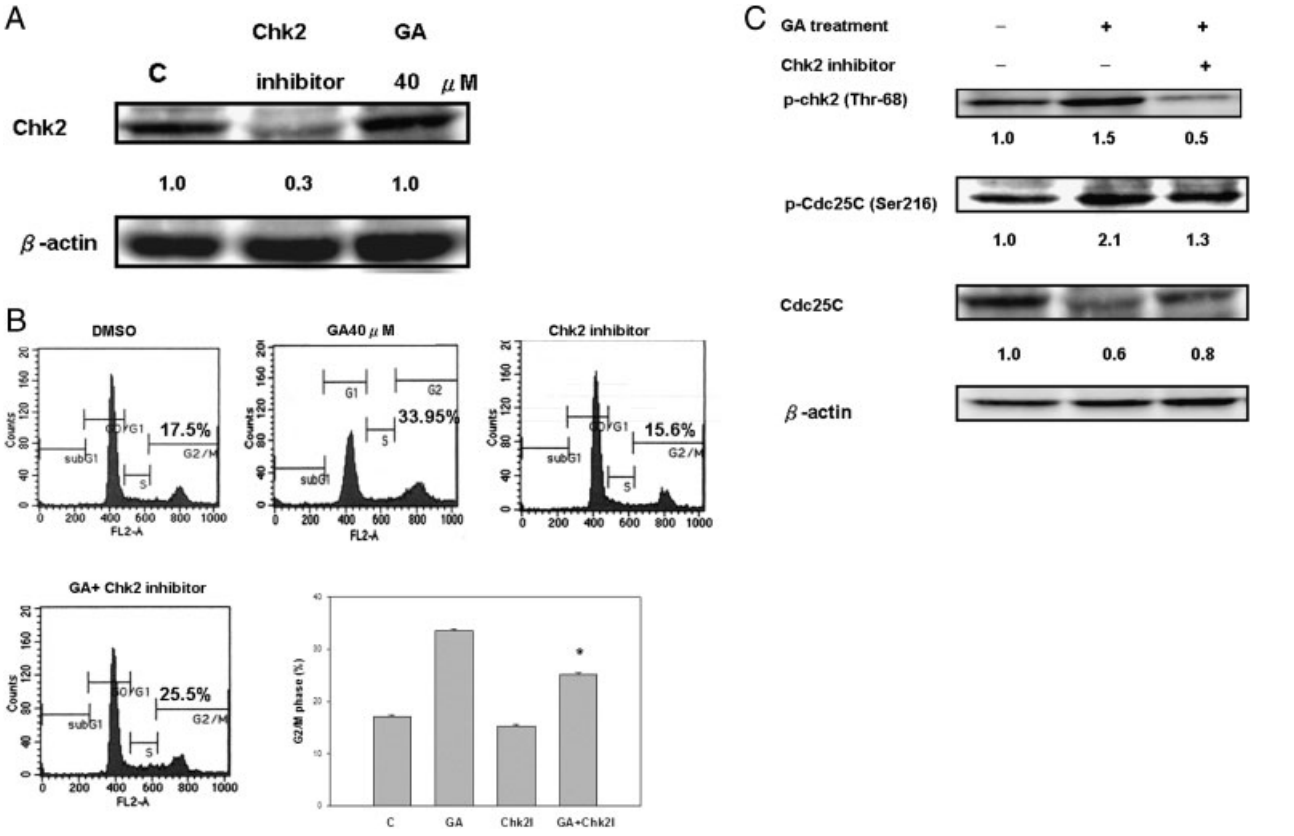

Figure 6. Effect of Chk2 inhibitor on down-regulation of Chk2 blocked GA-induced G2/M arrest. (A) Cells were treated with DMSO (control) and Chk2 inhibitor (20 μM) alone or 40 μM GA without or with 2-h pretreatment with chk2 inhibitor for 24 h. At the end of the treatment, total cells were collected and processed for Chk2 protein expression by western blotting. (B) Cell cycle analysis by flow cytometry or (C) lysate preparation as mentioned in Section 2. Cell lysates were analyzed for p-Chk2 (Thr-68), Chk2, p-Cdc25C (Ser-216), and Cdc25C protein levels by western blotting. Membranes were also stripped and re-probed with anti-β-actin antibody for protein loading control. The data shown in each case are mean ± SD of three independent samples and are representative of two independent experiments with comparable observations. $^{*}p < 0.01$.

in GA-treated (40 μM for 2 h) cells and is seen to be down-regulated by 24 h of treatment (Fig. 5).

3.6 Chk2 inhibitor-mediated down-regulation of Chk2 blocked GA-induced G2/M arrest

To experimentally verify the role of Chk2 in GA-induced cell cycle arrest, we used the specific Chk2 inhibitor to suppress Chk2 protein expression. As can be seen in Fig. 6A, cells treated with Chk2 inhibitor suppressed Chk2 protein expression by more than 70% and those treated with GA did not show a significant difference when compared with the control. Chk2 inhibitor-treated cells and control cells were then treated with GA, and their cell cycle distribution was assessed after 24 h (Fig. 6B). Reduction of Chk2 by its chemical inhibitor in the presence of GA treatment results in about a 1.6-fold increase in G2/M phase cells. The result shows that GA-induced G2/M block is partially but significantly attenuated in cells treated with Chk2 inhibitor.

The effect of Chk2 inhibitors on GA-induced phosphorylation of Chk2 and Cdc25C is also examined and the results are shown in Fig. 6C. The GA-induced Thr-68 phosphorylation of Chk2 is relatively more pronounced in the GA-treated cells than in Chk2 inhibitor-treated cells. Furthermore, GA-induced Ser-216 phosphorylation of Cdc25C is relatively more pronounced in GA-treated cells than in the cells treated with Chk2 inhibitor.

3.7 GA treatment promoted Ser-139 phosphorylation of histone H2A.X

H2A.X is a variant form of histone H2A that is directly phosphorylated at Ser-139 by an activated ATM kinase, making an early event in response to DNA damage, and is

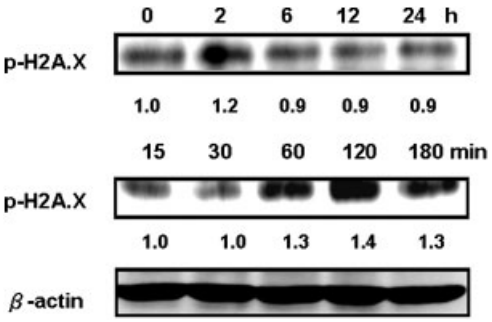

Figure 7. GA induces phosphorylation of H2A.X in TSGH-8301 cells. Cells were treated with DMSO (control) or 40 μM GA for the indicated time periods. At the end of the treatments, cell lysates were prepared and analyzed for p-H2A.X (Ser139) by western immunoblotting as mentioned in Section 2. Blots shown are representative of two independent experiments in each case.

also known to play a critical role in the retention of DNA repair factors at DNA-damaged sites [32]. We examined whether GA treatment caused DNA double-strand breaks by monitoring Ser-139 phosphorylation of H2A.X. Phosphorylation of H2A.X in GA-treated cells was clearly evident at the 2 h time point as determined by immunoblotting using phospho-specific H2A.X antibody (Fig. 7). We next conducted an early time point study; GA treatment of cells resulted in a strong phosphorylation of H2A.X as early as 1 h (Fig. 7). Altogether, these results indicated that GA treatment caused DNA double-strand breaks to activated ATM/Chk2.

4 Discussion

Plant polyphenols are secondary metabolites widely distributed in various groups of the higher plant kingdom. Many kinds of plant polyphenols, such as tannis and flavonoids, have been shown to be effective not only against tumor initiation, but also against the promotion phase of tumorigenesis [33–35]. These biological properties of plant polyphenols are now recognized as reasons why a polyphenol-rich diet or herbal medicines are effective in cancer prevention. Unchecked proliferative potential involving deregulation in cell cycle progression is generally described as a central process in the development of cancer [36]. In this regard, our findings of the present study suggest that GA exert strong antiproliferative effects against human bladder carcinoma TSGH-8301 cells, and this effect involves alterations in cell cycle regulators, causing G2/M arrest as well as cell death.

G2/M transition is regulated mainly by the sequential activation and deactivation of CDK-regulatory proteins and cyclin complexes [18, 37]. Cdc2 is also known as Cdk1, which initially forms a complex with Cyclin B1 to drive the cell from G2 to M phase. Cdc2 kinase activity is enhanced in many human cancers, allowing cell cycle progression of cancer cells and having oncogenic mutations in DNA for continued cell proliferation [18]. Therefore, induction of G2/M arrest in cancer cells is suggested as a potent approach to inhibit cell cycle progression and tumor growth. GA decreased Cdc2 and Cyclin B1 protein levels (Fig. 2), with a concomitant decrease in Cdc2 kinase activity in TSGH-8301 cells. These molecular alterations caused by GA could lead to a G2/M arrest in TSGH-8301 cells.

Cip1/p21 is a universal inhibitor of CDKs whose expression is normally regulated by the p53 tumor suppressor protein as well as by p53-independent mechanisms [38, 39]. Additionally, it plays a critical role in the cellular response to DNA damage for cell cycle arrest. Kip1/p27 is another member of CDKIs, which gets unregulated in response to antiproliferative signals for cell cycle arrest [40]. Our results showed that GA increase Cip1/p21 protein level (Fig. 3A) but not Kip1/p27 (data not shown). Therefore, the increased levels of Cip1/p21 might decrease CDK–cyclin kinase activity [41].

Cdc25 phosphatases control cell cycle progression by dephosphorylating and activating CDKs at positions Thr14 and Tyr15 [19]. Furthermore, Cdc25C dephosphorylates and activates Cdc2/cyclin B mitotic kinase complex and permits cell entry into mitosis [42]. In our study, GA decreased Cdc25C protein level in TSGH-8301 cells (Fig. 3B). These reports suggest that Cdc25C phosphatases are critical regulators of Cdc2–Cyclin B1 kinase activity. Furthermore, GA also increased the phosphorylation of Cdc25C at Ser-216, which is known to create a binding site for 14-3-3β proteins and export to the cytoplasm in the inactive form [20, 43]. Overall, the binding with 14-3-3β hinders nuclear accumulation of Cdc25C, which is required for activation of Cdc2–Cyclin B1 kinase in the nucleus [44, 45]. As GA-caused cell cycle arrest was not affected upon restoring the protein level of Cdc25C, cytoplasmic translocation of Cdc25C increasing the binding ability to 14-3-3β might be the main mechanism of cell cycle arrested by GA in our model (Fig. 4C).

Cdc25C phosphorylation is reported to be mediated *via* Chk2 kinase at Ser216 [20]. Genetic alterations of Chk2 have been identified in a wide range of human sporadic tumors, including carcinomas of breast, lung, vulva, ovary, and bladder [46, 47]. The present study indicated that GA treatment resulted in increased phosphorylation of Chk2 at Thr68 sites (Fig. 5). Downregulation of Chk2 using Chk2 inhibitor resulted in abolition of GA-induced G2/M arrest, as well as decreased the phosphorylation of Chk2 at Thr-68 and Cdc25C at Ser216 sites. Interestingly, we also observed a slight decrease in the Cdc25C protein level. Activation of Chk2 involves an initial phosphorylation step on Thr68 by ATM and plays a critical role in cellular response to DNA damage such as cell cycle arrest, DNA repair, and apoptosis [48]. Activation of ATM resulted in the phosphorylation of diverse downstream targets that involve in numerous cellular events, including regulation of cell cycle checkpoints (G1, intra-S, and G2-M) and apoptosis [48]. Consistent with these reports, GA treatment resulted in increased phos-

phorylation of ATM at Ser1981 (activated form), which could lead to the increased phosphorylation of Chk2 (Thr68) mediating G2-M cell cycle arrest. In addition to activating Chk1 and Chk2 for cell cycle arrest, ATM is known to phosphorylate a histone variant protein H2A.X at Ser139, making a double-strand DNA break and initiation of apoptosis [48]. We found that the increased phosphorylation of ATM (Ser-1981) as well as H2A.X (Ser139) indicated DNA double-strand breaks by GA in TSGH-8301 cells to decrease cell survival. However, the detailed mechanism in DNA damage remains for further investigation.

In summary, GA-induced G2/M cell cycle arrest involving molecular alterations in cell cycle regulatory proteins in human bladder carcinoma cells. G2/M arrest was mediated *via* decrease in the Cdc2 kinase activity, involving decrease in the protein levels of Cdc2 and Cyclin B1. The decreases in the levels of Cdc25C accompanied by an increased phosphorylation of Cdc25C (Ser216) resulting in its translocation to cytoplasm and binding with 14-3-3β could be a role in the G2-M arrest. Furthermore, the activation of upstream ATM leading to Chk2 activation (Thr68) and phosphorylation of H2A.X (Ser-139) would contribute to G2-M arrest and cell survival. The relative studies are in progress in our laboratories to assess the anticancer efficacy of GA and associated mechanism in different human TCC cells as well as in animal studies. The present results and the ongoing studies would provide scientific evidences in this ubiquitous dietary agent as a potent anticancer component in bladder cancer.

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