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Ethanol extract of loquat fruit skin inhibits the proliferation and metastatic potential of EJ human bladder carcinoma cells

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ABSTRACT

Although studies on biological properties of loquat plant have been performed, research on the molecular mechanism of action in loquat fruit skin is very limited. Therefore, we investigated the molecular mechanisms underlying the inhibitory activity of ethanol extract from loquat fruit skin (EEFS) against the proliferation and metastatic potential of EJ bladder carcinoma cells. Treatment of EJ cells with EEFS significantly inhibited their proliferation by inducing cell-cycle arrest at the G1 phase. Expression of CDK2, CDK4, cyclin D1, and cyclin E was altered by EEFS treatment. Interestingly, p27KIP1, but not p21WAF1 or p53, was identified as a candidate CDK inhibitor (CDKI) associated with the EEFS-mediated inhibition of proliferation. Among mitogen-activated protein kinases, ERK1/2 phosphorylation was significantly elevated but not that of p38 and JNK. In addition, phospho-AKT (protein kinase B; PKB) was not affected by EEFS treatment. Aggressive phenotypes, including migratory and invasive potentials of EJ cells, were similarly diminished by EEFS. Gelatin zymography showed that the enzymatic activity of matrix metalloproteinase (MMP)-9 was markedly inhibited by treatment with EEFS. Results from electrophoretic mobility shift assays demonstrated that MMP-9 inhibition was associated with reduced binding activities of transcription factors NF- κ B, activator protein-1, and specificity protein 1 to its promoter region. Taken together, our data clearly indicate that EEFS inhibits the proliferation of EJ cells through p27KIP1, which is associated with G1 arrest and increased extracellular signal-regulated kinase phosphorylation. Metastatic potential represented by MMP-9 activity was also remarkably inhibited by EEFS, suggesting that EEFS is a novel chemotherapeutic agent for bladder cancers.

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Introduction

Bladder cancer, typically transitional cell carcinoma (TCC), is one of the most common cancers among men (Siegel et al. 2017). TCC is classified into two subgroups: non-muscle-invasive superficial cancer and muscle-invasive cancer. Superficial cancer accounts for approximately 70% of all bladder cancer cases and is manageable through a transurethral resection of the bladder tumor (Raghavan 2003). However, superficial bladder cancers at advanced stages or muscle-invasive carcinomas are often life-threatening due to their invasiveness to other tissues or organs. Therefore, developing novel chemo-preventive agents is of critical importance to increase the long-term survival rate of cancer patients.

Cancer cells respond to treatment with chemo-preventive reagents through modulation of kinase activities in key signaling pathways, including mitogen-activated protein kinases (MAPKs) and PI-3K/AKT (Gerhardt et al.

2014; Zheng et al. 2014). These molecular signals, eventually transferred to the nucleus of the cell by signaling cascades of the effectors, modulate activities of regulators associated with the cell cycle, including cyclin-dependent kinases (CDKs) and cyclins at specific cell-cycle phases (G1, S, and G₂/M) (Lee and Yang 2001; Li and Blow 2001). Among cell-cycle checkpoints, the G1-checkpoint has been considered a critical target for chemotherapeutic agents (Lin et al. 1998; Li et al. 2004). The progression of G1 to S phase is commonly determined by the formation of protein complexes between cyclin D1/CDK 4 and cyclin E/CDK2 (Sherr 1996). These kinases are often negatively regulated by the expression of CDKIs, including p21WAF1 and p27KIP1, which were reported to be up-regulated by some chemo-preventive reagents (Izutani et al. 2012; Chen et al. 2016). In addition, evidence suggests that matrix metalloproteinases (MMPs), including MMP-9,

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correlate with the onset and progression of bladder cancers, which is associated with the activation of activator protein-1 (AP-1), specificity protein 1 (Sp1), and nuclear factor- κ B (NF- κ B) transcription factors (Davies et al. 1993; Bianco et al. 1998).

Loquat (*Eriobotrya japonica*), belonging to the family of Rosaceae, is an evergreen flowering plant that is indigenous to southeastern China. The loquat fruit has been utilized for a long time as a traditional medicine for asthma and has drawn increasing attention in recent years due to its diverse biological activities including anti-inflammatory (Choi et al. 2011), anti-diabetic (Tanaka et al. 2008), anti-cancer (Alshaker et al. 2011), and anti-oxidative activities (Zhou et al. 2011). Kim et al. reported that loquat methanol extracts showed significant inhibitory activity against invasiveness of MDA-MB-231 human breast cancer cells by inhibiting enzymatic activities of MMPs (2009). From the same research group, You and coworkers examined the inhibitory effect of extracts from loquat leaves *in vivo* using xenografted mice inoculated with MDA-MB-231 human breast cancer cells and demonstrated that the inhibitory activity of loquat leaves was associated with downregulation of both ERBB3 and MMPs (You et al. 2016).

Although biological activities of the loquat plant have been intensively studied for decades, research on the anti-cancer effect of loquat fruit skin is very rare. In this study, we investigated the anti-cancer activity of ethanol extracts from loquat fruit skin in the proliferation, migration, and invasion of bladder cancer EJ cells. In addition, to better understand the mode of action of loquat extracts, we identified key molecular effectors in the loquat-induced inhibition of cellular responses.

Materials and methods

Materials

For details about antibodies and chemicals utilized in the study, please refer to the supplementary information.

Preparation of ethanol extracts from loquat fruit skin

The skins of the loquat fruit were finely crushed and dried. Dried samples (10 g) were soaked in 100 ml of 50% ethanol and mixed in a shaker at 170 rpm at room temperature for 24 h. The extracts were filtered and evaporated via a rotary evaporator at below 50°C, lyophilized, and freeze-dried. The yields of final extracts were approximately 10% w/w, and were diluted in ethanol to produce ethanol extracts from loquat fruit skin (EEFS).

Cell counting

Fifty microliters of trypsinized cells was mixed with 50 μ l of 0.4% trypan blue (Sigma-Aldrich, MO, USA). Twenty microliters of cell suspension was loaded into a hemocytometer and counted in triplicate.

MTT cell proliferation assay

Proliferation of EJ cells was measured with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Briefly, 2×10^3 cells were seeded in 96-well plates followed by the treatments as indicated. Cell density was evaluated by absorbance at 490 nm. Experiments were performed in quadruplicate.

Fluorescence-activated cell sorting cell-cycle analysis

Cells (2×10^6) were plated in 100-mm culture plates and treated with EEFS (0–800 μ g/ml) for 24 h. Then, cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Cell pellets were resuspended and fixed with 70% ice-cold ethanol followed by incubation for 20 min at 4°C. Fixed cells were washed twice with cold PBS and resuspended in 500 μ l of PBS. Cells were treated with RNase (1 mg/ml) followed by propidium iodide (50 mg/ml). Phase distribution of the cell cycle was measured with a FACStar flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with BD Cell Fit software.

Immunoblots and immunoprecipitation

Protein lysates were prepared as described previously (Lee et al. 2013). Protein lysate was separated by electrophoresis on a 10% polyacrylamide gel under denaturing conditions. For more details, please refer to the supplementary information.

Wound-healing migration and Boyden chamber invasion assays

Cells (3×10^5 per well) were seeded in 6-well plates. In order to inhibit cell proliferation, EJ cells were pre-incubated with 5 μ g/ml mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. For more details, please refer to the supplementary information.

Gelatin zymography

Upon reaching approximately 90% confluency, EJ cells were treated with EEFS (0–800 μ g/ml) for 24 h.

The culture medium was collected and separated by SDS-PAGE through a polyacrylamide gel containing 1 mg/ml gelatin. The gels were washed with 2.5% Triton™ X-100 for 2 h at room temperature, followed by incubation in a buffer containing 150 mM NaCl, 10 mM CaCl₂, and 50 mM Tris-HCl (pH 7.5) at 37°C overnight. The gels were then stained with Coomassie blue (0.2%) and photographed on a light box. Gelatin-degrading enzymatic activities of MMPs were measured as the size of white zones.

Nuclear extract and electrophoretic mobility shift assay

After collecting EJ cells by centrifugation, cells were washed and resuspended in 1× HEPES buffer and nuclear extracts were collected. Nuclear extracts (10–20 µg) were pre-incubated with 100-fold excess unlabeled oligonucleotides spanning the –79 position of the MMP-9 cis-element of interest at 4°C for 30 min. For details including the oligonucleotide sequences and procedures, please refer to the supplementary information.

Statistical analysis

Data are represented as mean ± SD. Data were compared by factorial ANOVA and a Fisher's least significant difference test, where appropriate. Differences were considered statistically significant at $P < .05$.

Results

EEFS inhibits proliferation of EJ bladder cancer cells in a dose-dependent manner

First, we investigated whether EEFS affects the proliferation of EJ cells. After treating the cells with EEFS (0–800 µg/ml) for 24 h, cellular viability was measured by both a viable cell counting method (Figure 1(A)) and an MTT assay (Figure 1(B)). As demonstrated by the viable cell counting result, treatment with EEFS significantly reduced the proliferation of EJ cells in a dose-dependent manner (Figure 1(A)). MTT assays verified the dose-dependent suppressive activity of EEFS (Figure 1(B)). In addition, EJ cells acquired a rounded morphology after treatment with EEFS, which may indicate the development of apoptosis (Figure 1(C)).

EEFS treatment induces G1-phase cell-cycle arrest

In order to examine which cell-cycle phase is associated with the EEFS-induced growth inhibition in EJ cells, we investigated the cell-cycle phase distribution of EJ cells treated with EEFS (0–800 µg/ml) for 24 h using flow cytometric analysis. Treatment with EEFS at 0, 400, 600, and 800 µg/ml resulted in a dose-dependent accumulation of EJ cells in the G1 phase (Figure 2(A–E)). After treatment with 800 µg/ml EEFS, accumulation of the cells in the G1 phase increased up to approximately 60% compared with that of the control (Figure 2(E)). Therefore, these

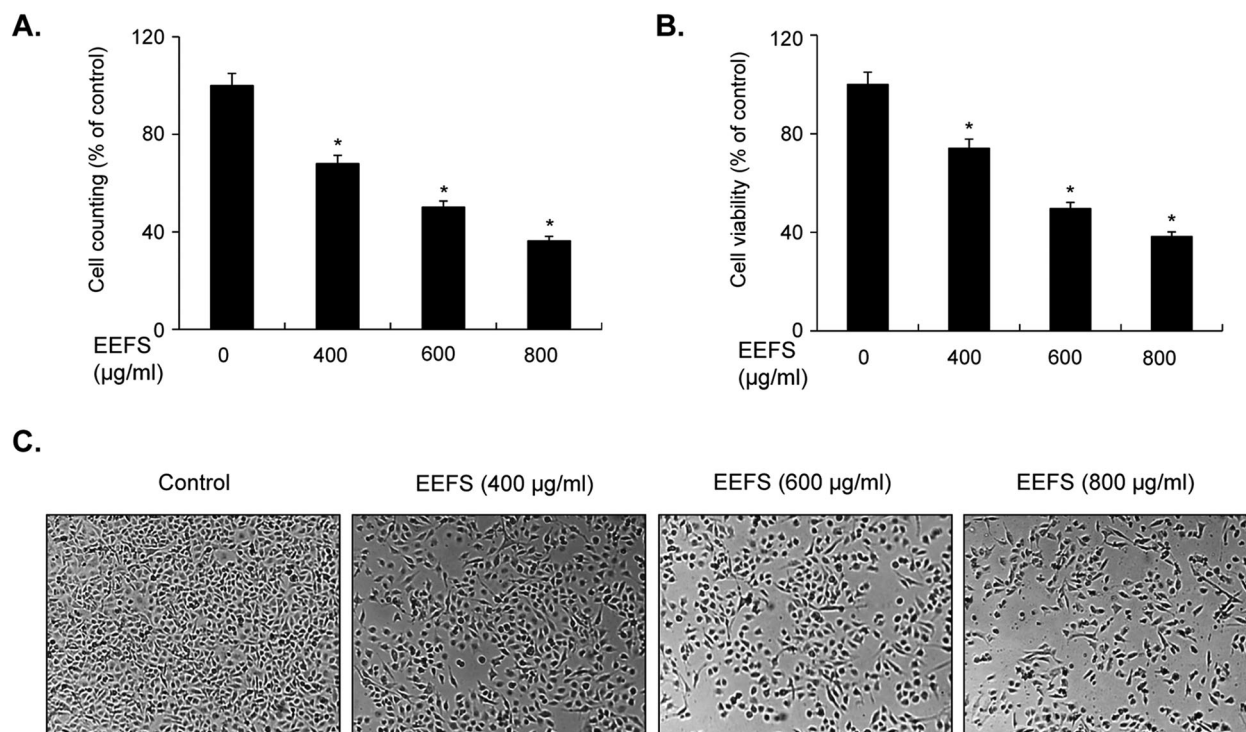


Figure 1. Inhibition of proliferation of EJ bladder cancer cells by EEFS.

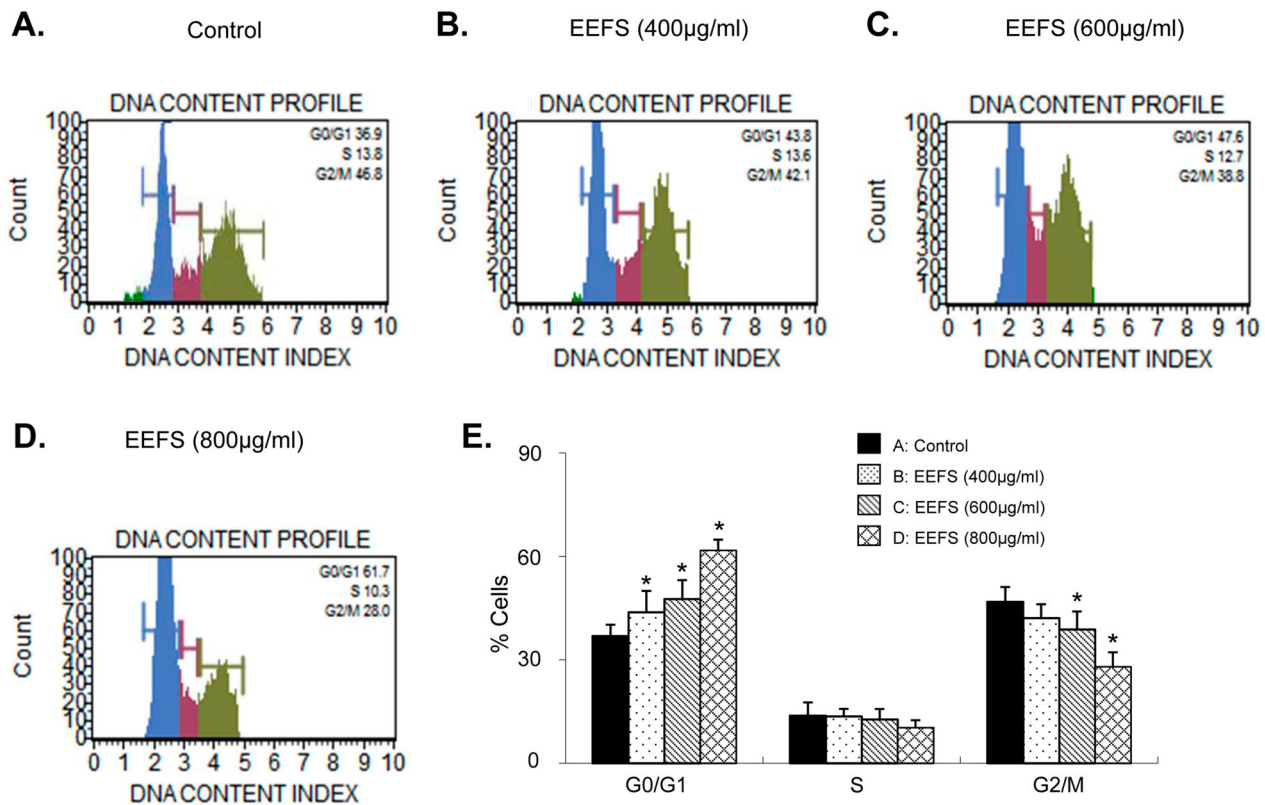


Figure 2. Cell-cycle phase distribution of EJ cells treated with EEFS.

data indicate that EEFS inhibits the proliferation of EJ bladder cancer cells via induction of G1 cell-cycle phase arrest.

EEFS induces G1-phase cell-cycle arrest via increased expression of p27KIP1 and downregulation of cyclin/CDK complexes

Proliferation of mammalian cells is tightly regulated by molecular association of cyclins and CDKs. When the cells are damaged, cell-cycle progression is limited by cyclin-dependent kinase inhibitors such as p21WAF1 and p27KIP1, which inhibit kinase activities of CDKs. In order to understand the mode of action of EEFS, we treated EJ cells with EEFS (0, 400, 600, and 800 µg/ml) and then examined the expression of protein effectors associated with the regulation of the G1 cell-cycle phase. Among regulators, CDK2, CDK4, cyclin D1, and cyclin E were downregulated by EEFS treatment in a dose-dependent manner (Figure 3(A)). Interestingly, together with the reduced expression of cyclins and CDKs, expression of p27KIP1 was remarkably increased by EEFS treatment dose-dependently (Figure 3(B)). However, expression levels of p21WAF1 and p53 were unchanged. In order to further understand the role of p27KIP1 in EEFS-mediated induction

of G1-arrest of EJ cells, we carried out immunoprecipitation for EEFS-treated EJ cells using anti-CDK2 and anti-CDK4 antibodies followed by immunoblots with p27KIP1 antibody. As shown in Figure 3(C), the association of p27KIP1 with CDK2 and CDK4 was markedly increased by EEFS treatment. This result clearly demonstrates that EEFS inhibits the proliferation of EJ cells by up-regulating expression of p27KIP1, which consequently inhibits the expression of CDK 2 and CDK 4.

EEFS increases phosphorylation of extracellular signal-regulated kinase (ERK) in EJ bladder cancer cells

Several lines of evidence suggested that the MAPK (ERK1/2, JNK1/2, and p38) and PI3K-AKT signaling pathways contribute to the progression of bladder cancers (Dangle et al. 2009; Gerhard et al. 2014). Therefore, we investigated protein expression of MAPKs and AKT in EEFS-treated EJ cells. As shown in Figure 4, treatment with EEFS (0, 400, 600, and 800 µg/ml) led to increased phospho-ERK levels in EJ cells (Figure 4). At 800 µg/ml of EEFS, phospho-ERK increased 7.3-fold compared with that of the control. However, changes in phospho-p38, phospho-JNK, and phospho-AKT levels were negligible (Figure 4). These data suggest that ERK signaling

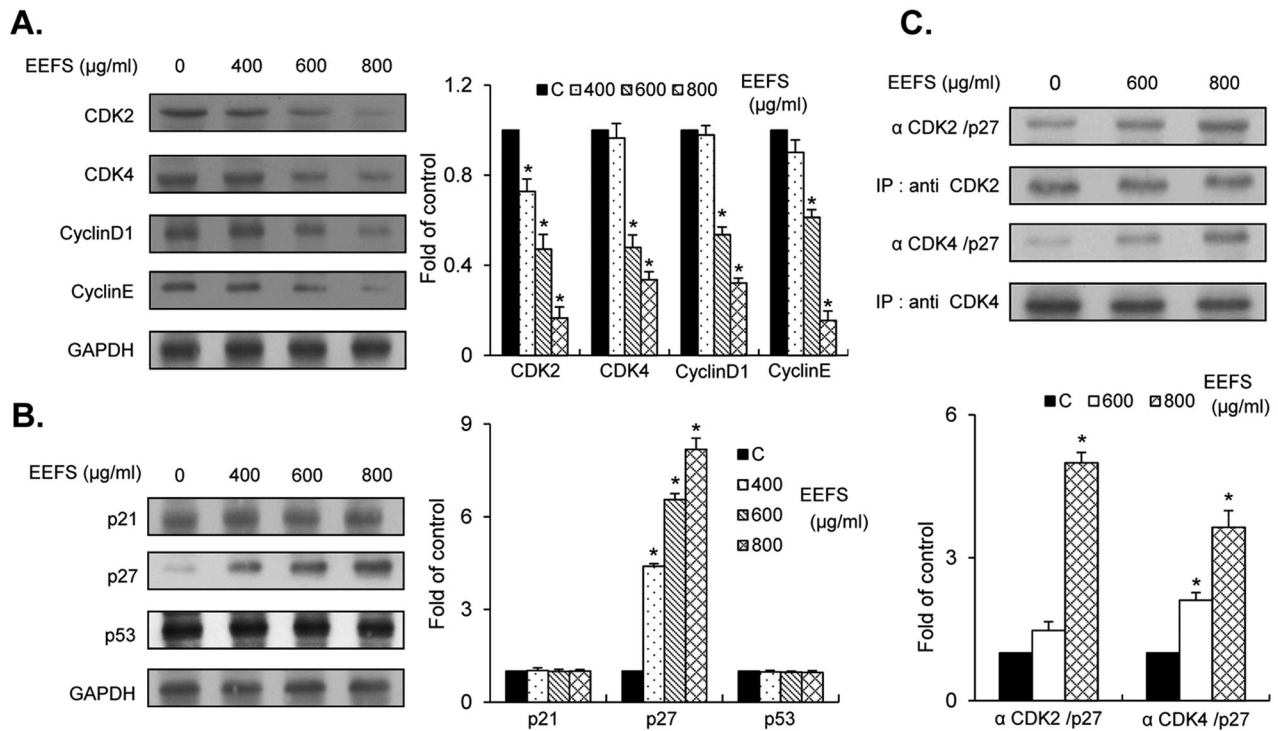


Figure 3. Cell-cycle regulators in EEFS-treated EJ cells.

is involved in EEFS-induced inhibition of EJ cell proliferation.

EEFS reduces the migratory capacity and invasiveness of EJ bladder cancer cells

Bladder cancer cells at advanced stages often acquire metastatic potential through enhanced migratory and invasive capacities. Therefore, we investigated the influences of EEFS on the migration and invasion of EJ cells

by *in vitro* wound-healing migration and invasion assays. In order to exclude the influence of proliferation, EJ cells were pre-incubated with mitomycin C (5 µg/ml) for 2 h. As shown in Figure 5(A), the migration of EJ cells was markedly inhibited by EEFS treatment compared with the migration of control cells. The effect of EEFS on the invasiveness of EJ cells was also assessed using Boyden chamber assays. As shown in Figure 5(B), invasiveness of EJ cells was significantly diminished by treatment with EEFS. The number of cells transgressing

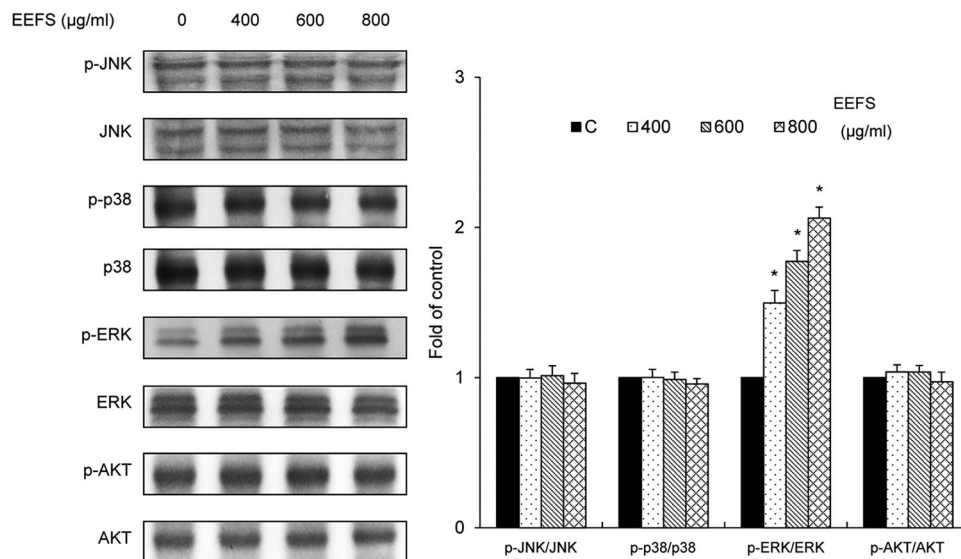


Figure 4. Changes of phosphorylation of MAPKs and AKT in EEFS-treated EJ cells.

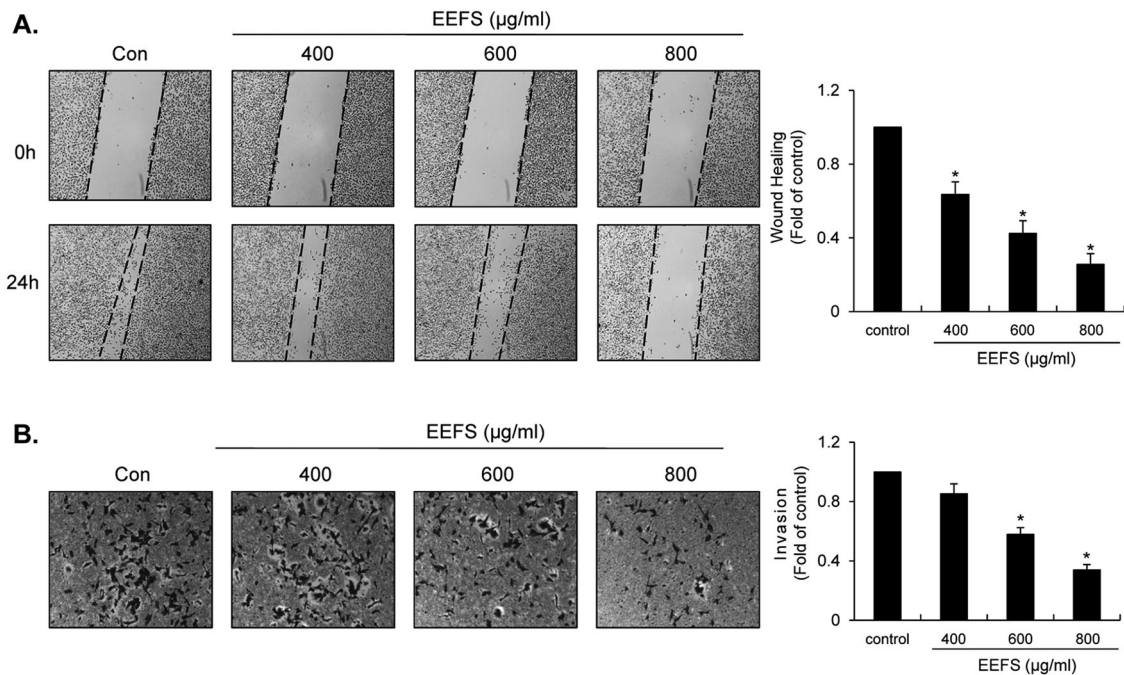


Figure 5. Inhibition of migration and invasion of EJ cells induced by EEFS.

the transwell membrane was reduced by approximately 70% by treatment with 800 µg/ml EEFS. These results indicate that EEFS effectively inhibits the migratory and invasive potential of EJ bladder cancer cells.

EEFS inhibits gelatinase activity of MMP-9 through suppression of binding capacities of transcription factors, NF-κB, AP-1, and Sp1 in EJ cells

Over-expression of enzymes degrading the extracellular matrix is an intrinsic characteristic of transformed cells. In bladder cancers, MMP-type proteolytic enzymes have been reported as critical determinants for metastatic potential of the neoplastic disease (Bianco et al. 1998; Dano et al. 1999). Thus, we examined whether EEFS inhibits enzymatic activities of MMP-2 and -9 using gelatin zymography. As shown in Figure 6(A), treatment with EEFS significantly suppressed gelatinase activities of MMP-2 and -9. The enzymatic activities of both MMP-2 and -9 were reduced more than 80% after treatment with 800 µg/ml EEFS (Figure 6(A)). To understand the molecular mechanism of EEFS-mediated suppression of enzymatic activity of MMP-9, we performed electrophoretic mobility shift assays (EMSAs) using oligonucleotides of corresponding transcription factor response elements (i.e. NF-κB, AP-1, and Sp1) that are located in the MMP-9 promoter region. EJ cells were treated with 600 and 800 µg/ml EEFS for 24 h, and then were subjected to EMSA assays. The binding capacities of NF-κB, AP-1, and Sp1 to the MMP-9 promoter fragment were remarkably reduced in a dose-

dependent manner (Figure 6(B)). These results clearly indicate that EEFS significantly inhibits the migratory and invasive potential of EJ cells by suppressing MMP-9 activity via reduction of binding activities of the transcription factors, NF-κB, AP-1, and Sp1.

Discussion

Traditionally, loquat has been considered a medicinal plant in the southeastern region of China due to its diverse biological activities. Although the loquat plant has been reported to possess health benefits to the public, research on extracts from the skin of loquat fruit is quite rare. Therefore, in this study, we investigated the molecular mechanism of the inhibitory activity of EEFS against the proliferation and invasive potential of bladder cancers, using EJ bladder carcinoma cells as a model system.

First, we observed that EEFS significantly inhibited the proliferation of EJ cells with morphological changes. Similar to our study, Banno et al. reported that solvent extracts from loquat leaves significantly inhibited the growth of skin tumors induced by carcinogenic stimuli in mice (2005). Kikuchi et al. investigated the anti-cancer activity of extracts from loquat leaves against human leukemia cell lines and identified that 3-O-(E)-p-coumaroyl tormentic acid exhibited the strongest activity in inducing caspase-dependent apoptotic cell death among the compounds extracted (2011). Since effectors of the G1 cell-cycle phase are frequently mutated in tumorigenesis,

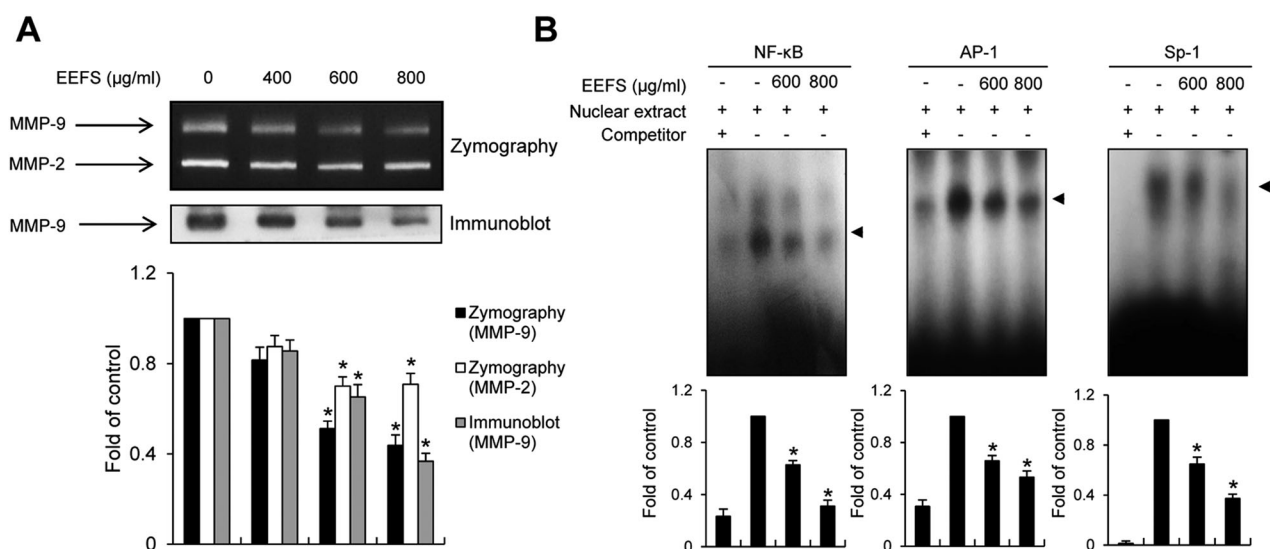


Figure 6. EEFS inhibits enzymatic activity of MMP-9 via suppression of binding activities of transcription factors, NF- κ B, AP-1, and Sp1.

many chemotherapeutic reagents, including tamoxifen, paclitaxel, and 5-fluorouracil, target the G1 phase of the cell cycle (Osborne et al. 1983; Lin et al. 1998; Li et al. 2004). Uto et al. reported that triterpenes derived from *E. japonica* exhibited anti-proliferative activity against leukemia cell lines via accumulation of the cells at the sub-G1 phase (2013). In our study, the cell-cycle distribution analysis of EEFS-treated EJ cells showed that EEFS led to a cell-cycle arrest at the G1 phase. Based on these results, we investigated the levels of protein effectors altered by EEFS treatment. In mammalian cells, the G1-to-S phase transition is frequently determined by the interactions between cyclins (cyclin D1 and cyclin E) and CDKs (CDK2 and CDK4) (Li and Blow 2001; Owa et al. 2001). We showed that EEFS treatment led to downregulation of CDK2 and CDK4 levels and their associated partners, cyclin D1 and cyclin E. Subsequently, levels of p27KIP1 were upregulated by EEFS treatment but not those of p21WAF1 or p53. Pull-down assays using antibodies of CDK2 and CDK4 suggested that complexes of CDKs/cyclins are indeed downregulated by the CDKI, p27KIP1. Taken together, these results clearly demonstrate that EEFS exhibits anti-proliferative activity against bladder cancer cells via G1 cell-cycle phase arrest.

The roles of MAPKs and PI3K-AKTs in cell-cycle regulation have been well established in many studies (Chang et al. 2003; Chambard et al. 2007; Vadlakonda et al. 2013). Results from our study showed that ERK1/2 phosphorylation was closely associated with the mode of action of EEFS. Indeed, phospho-ERK1/2 was remarkably increased by EEFS treatments. In general, ERK1/2 has been reported as an effector associated with cellular proliferation (Johnson and Lapadat 2002). However, in some cases, activation of ERK1/2 equally leads to

apoptotic cell death in cancer cells. The ERK1/2-mediated apoptotic responses have been reported especially in chemotherapeutic reagents including cisplatin, doxorubicin, and etoposide (Woessmann et al. 2002; Park et al. 2012; Shin et al. 2016). Kim and Shin reported that aqueous extracts from leaves of loquat plants showed anti-inflammatory activity in HMC-1 human mast cells by suppressing the phosphorylation of p38 and ERK, but not of JNK (2009). These results indicate that cellular responses to extracts from loquat plants may be cell type-dependent.

In addition, EEFS significantly inhibited the malignancy of EJ cells via suppressing migratory and invasive activities in a dose-dependent manner. These results were associated with the inhibition of enzymatic activities of MMPs (MMP-2 and MMP-9) as demonstrated by gelatin zymography. Similar to our results, Kim et al. reported that loquat extracts suppressed the adhesion, migration, and invasion of human breast cancer cells by inhibiting enzymatic activities of MMP-2 and MMP-9 (2009). Cha et al. reported similar results where the ethyl acetate fraction of the leaves of *E. japonica* significantly suppressed the activities of MMP-2 and MMP-9 in B16F10 mouse melanoma cells (2011). These results demonstrate that EEFS plays an inhibitory role in the metastatic potential by down-regulating the activity of MMP-2 and MMP-9.

Since previous studies have demonstrated that MMP-9 is deeply associated with invasive bladder cancer progression (15, 16, 27), our next step was to focus on MMP-9 regulation in EEFS-treated EJ cells. To do this, we investigated which transcription factors were involved in the suppression of MMP-9 activity. Kwon et al. revealed that methyl chlorogenic acid extracted

from leaves of *E. japonica* had strong inhibitory activity against NF- κ B in mouse liver cells *in vivo* (2000). Utilizing EMSA assays, we found that NF- κ B, AP-1, and Sp1 transcription factors could participate in the suppression of MMP-9 induced by EEFS. These studies indicate that reduced MMP-9-mediated transcription factors might be involved in the inhibition of migration and invasion in EEFS-treated bladder cancer EJ cells.

In conclusion, these results suggest that EEFS possesses a strong anti-cancer activity against the proliferation, migration, and invasion of EJ bladder cancer cells through G1 cell-cycle arrest, increased phosphorylation of ERK1/2, and inhibition of MMP-9 via suppression of binding of NF- κ B, AP-1, and Sp1 transcription factors. These data strongly suggest the need for intensive studies on loquat fruit skin, especially *in vivo*, as an inhibitor of tumor proliferation and metastasis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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