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Anti-Proliferative Effects of Polyphenols from Pomegranate Rind (*Punica granatum* L.) on EJ Bladder Cancer Cells Via Regulation of p53/miR-34a Axis

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miRNAs and their validated miRNA targets appear as novel effectors in biological activities of plant polyphenols; however, limited information is available on miR-34a mediated cytotoxicity of pomegranate rind polyphenols in cancer cell lines. For this purpose, cell viability assay, Realtime quantitative PCR for mRNA quantification, western blot for essential protein expression, p53 silencing by shRNA and miR-34a knockdown were performed in the present study. EJ cell treatment with 100 μg (GAE)/mL PRE for 48 h evoked poor cell viability and caspase-dependent pro-apoptosis appearance. PRE also elevated p53 protein and triggered miR-34a expression. The c-Myc and CD44 were confirmed as direct targets of miR-34a in EJ cell apoptosis induced by PRE. Our results provide sufficient evidence that polyphenols in PRE can be potential molecular clusters to suppress bladder cancer cell EJ proliferation via p53/miR-34a axis. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: anti-proliferative effects; p53; miR-34a; pomegranate rind.

INTRODUCTION

Consumption of fruits and vegetables in diet with abundant polyphenols has been confirmed to reduce oxidant stress (Veskoukis *et al.,* 2012), inflammatory (Biasi *et al.,* 2011) and cancer cell invasion (Karna *et al.,* 2011) in many studies. In some berries, numbers of polyphenol chemical characteristics and their antioxidant effects have been systematically studied in past years. Recently, with the increasing interest of tumor chemotherapy research, the suppressing effect of natural polyphenols from berries on carcinoma cell proliferation has been constantly revealed *in vivo* and vitro (Scholtysek *et al.,* 2009; Seeram, 2008).

Pomegranate (*Punica granatum* L.) was a popular berry fruit planted all over the world. Anti-microbial (Gould *et al.,* 2009), reducing obesity (Al-Muammar and Khan, 2012) and activities against other human diseases of pomegranate extracts from several kind of parts, such as flowers, leaves and seeds, were demonstrated in many literatures. Moreover, several studies were reported on the anti-proliferative and apoptosis ability of pomegranate extracts against several tumor cells (Syed *et al.,* 2013). Though pomegranate rind was the agricultural by-products of pomegranate, polyphenols in pomegranate rind were thought to be homologous to the edible part of pomegranate. Therefore, it is consequential to efficiently utilize and explore the natural anti-tumor agents in pomegranate rind, especially the essential component, polyphenols.

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Transitional cell carcinoma is the most typical carcinoma and accounted for most of bladder tumors (Pignot *et al.,* 2012). The tumor suppressor gene p53 is encoded by TP53 gene and protecting bodies against the accumulation of mutated proto-oncogenes for the progression of cancer (Lopez *et al.,* 2012), which is essential for cell cycle arrest and apoptosis (Li and Johnson, 2013). In bladder cancers, p53 is recognized to be abnormally inactivated in more than 50% of carcinogenesis (Hilton and Svatek, 2012), which results in deterioration of cancer. Generally, c-Jun is considered a kind of proto-oncogenes and maturating the AP-1 early response transcription factor through binding with c-Fos (Chen and Tan, 2000). Some cell cycle proteins, such as c-Myc and cyclin D1 not only promoted cell survival, proliferation and tumorigenesis, but also inhibited tumor cell apoptosis by cooperating with c-Jun. In addition, c-Jun is the direct upstream element of p53 regulating cell cycle distribution (Skopelitou *et al.,* 1997), which is involved in the function of micro-RNAs (miRNAs) on intensifying mRNA degradation or blocking translation of candidate tumor-survival genes (Ferreira *et al.,* 2014).

With the clarity of biological function of miRNAs, the complexity of gene regulation in the cancer cells has been a challenge but also an opportunity for cancer chemotherapy because miRNAs could be candidate targets of specific lead compounds. A sufficiently identified microRNA, miR-34a, was found to be a direct target of p53 and subsequently mediated effects on cell cycle and apoptosis regulation (Hermeking, 2009). Likewise, recently increasing numbers of achievements indicated that natural polyphenols could mediate the target gene silencing via modulating miRNAs level in cancer cells (Banerjee *et al.,* 2012). Therefore, the aims of this study

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are to estimate anti-proliferative and pro-apoptosis effects of polyphenols from pomegranate rind on EJ bladder cancer cells. The functional mechanism of p53/miR-34a axis on EJ cell apoptosis induced by polyphenols of pomegranate rind was also explored.

MATERIALS AND METHODS

Reagents. Standards for identification and quantitative analysis of high-performance liquid chromatography spectrometry (HPLC) were obtained from Sigma-Aldrich (St Louis, MI). Primary antibodies against c-Myc, CD44, c-Jun, p53, cleaved caspase-3 p38-MAPK, phospho-p38 (Thr180/Tyr182), Bcl-2 and cleaved PARP were purchased from Cell Signaling Technology (Beverly, MA). For quantification of total phenolic, Folin–Ciocalteu reagent and gallic acid (GA) were purchased from Sigma-Aldrich (St. Louis, MO, US). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific. mirVana™ extraction kit was purchased from Applied Biosciences (Foster City, CA). ReverTra Ace (Toyobo), RQ1 RNase-Free DNase (Promega), FastStart Universal SYBR Green Master (Roche) and T4 DNA Ligase (Promega) were used for reverse transcription, quantification of mi-RNA/mRNA target and vector contraction, respectively. AntagomiR of miR34a and their non-special control of each were purchased from Ambion® (Life Technologies, US). All other chemicals and reagents were of analytical or biochemical grade, unless specified.

Preparation of the total polyphenols from pomegranate rind. The pomegranate (*Punica granatum* L.) was purchased from the Shahu fruit market (Wuhan, China). A voucher specimen Pomegranate rind (BH-005) was authenticated by Professor Hong Zhang (Department of Pharmacy, Renmin Hospital of Wuhan University). Pomegranate rind extract (PRE) was prepared and standardized to contain 13% *w/w* ellagic acid according to the previous literature (Panichayupakaranant *et al.,* 2010). The pomegranate rind was dried at 60 °C for 24 h in a drying oven and then smashed to powder using a grinder. The dried powder of pomegranate rind (1 kg) was extracted twice with 4 L of 90% (*v/v*) methanol aqueous solution under reflux conditions for 1 h. The pooled extracts were vacuum desiccated. The dried products were then suspended in 2% aqueous acetic acid and extracted with 400-mL ethyl acetate for 4 times. The product of ethyl acetate fraction was obtained by vacuum rotary evaporation. Total phenolic content in PRE was determined by the Folin–Ciocalteu assay and expressed as the percentage of gallic acid equivalents (GAE) in PRE (*w/w*). The μg/mL used in this study is the GAE of polyphenols in PRE.

HPLC-MS analysis of PRE. HPLC-MS was performed to quantitate polyphenolic composition including ellagic acid, punicalins, punicalagin A and B in PRE on a ZORBAX Eclipse Plus C18 column (Agilent Technologies, US, 250 mm × 4.6 mm, 5 μm) at 35 °C. A mobile phase of water with 0.1% (*v/v*) TFA was run in Phase A, and methanol was run in Phase B. A gradient program for 10-μL PRE methanol solution ran at 1 mL/min initially as follow: 0–5 min, 92%–70% of A; 5–11 min, 70% of A; 11–11.5 min, 70%–55% of A; 11. 5–30 min, 55% of A. Detection was at 254 nm for punicalins, punicalagin A, punicalagin B and ellagic acid. The pomegranate polyphenolics were analyzed in negative ESI-MS under the following conditions: sheath gas (N2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V.

Cell culture and viability assay. Human bladder carcinoma EJ cell line was an invasive, poorly differentiated transitional cell carcinoma of the bladder and purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin at 37 °C with 5% CO_2 atmosphere. Normal rat urinary bladder epithelial cells (RUBE cells) were isolated and maintained as previously described (Pauli *et al.,* 1980).

Cytotoxicity of PRE on EJ and RUBE cells was assessed by utilizing a water-soluble tetrazolium salt, WST-8 (also known as Cell Counting Kit-8). Briefly, cells were seeded in 96-well plate by 80% fusion and treated with different contents of PRE from 0 to 200 μg/mL at 24 h, 48 h and 72 h, respectively. Following administration, cells were washed with fresh medium and incubated with 10-μL WST-8 solutions for 1 h. The amount of the formazan dye, which is directly proportional to the numbers of viable cells, was measured at 450 nm by Multiskan FC plate reader and analyzed with Skanlt for Multiscan FC software (Thermo Scientific). The cell viability of PRE-treated cells was normalized to the vehicle-treated group (Control). In effect of caspase-3, -8 and -10 activation on EJ cell survival, cells were preferentially incubated in the absence or presence of Z-AEVD-FMK (20 μM), Z-IETD-FMK (20 μM), Z-DETD-FMK (20 μM), Z-VAD-FMK (20 μM) for 4 h before PRE treatment, respectively.

Evaluation of miRNAs and mRNA expression levels by quantitative reverse transcription PCR. After treatment with PRE, total RNA of cells in 6 well plates was extracted using the mirVana™ miRNA Isolation Kit (Invitrogen) according to the manufacturer's recommendation. For mRNA expression assay, 1 μg of mRNAs of each sample was reverse transcripted to complementary DNA (cDNA) with oligo (dT) 18 primer as previously stated. Instead of oligo (dT), 50-nM specific stem-loop RT primer (5′-GCA GCG AAT CCA CGA TTA GAA CAA CCA G-3′) for miR-34a was employed for the reaction of converting miRNA to cDNA. The products of RT were stored at −80 °C for further use.

The quantitative real-time RT-PCR (qRT-PCR) was performed a MiniOpticon™ (Bio-Rad, Hercules, CA, USA) using FastStart Universal SYBR Green Master (Roche, USA); the thermal cycling condition for PCR was 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 55 °C for 12 s, and 55 °C for 12 s, followed by 72 °C for 10 min. The primers used in real-time PCR are provided in the Supplemental materials.

Transfection with antagomiR of miR-34a. AntagomiR is a small synthetic RNA, which specifically knocks down the miRNA expression, sequentially regulating the expression of many interoperable mRNA molecules of the miRNA. In this study, EJ cells were seeded in 6-well plates and cultured for 80% fusion. Twenty nanometers of antagomiR of miR-34a or its scrambled control was transfected into cells for 6 h, respectively. The transfection mix was replaced by the medium in the presence or absence of PRE. The total-RNA and protein were isolated 48 h post treatment for further study.

Western blotting. After treatment, cells were harvested and lysed in M-per regent (Pierce, Rockford, IL). Protein concentration was determined by the BCA assay. Thirty micrograms of protein samples was separated by SDS PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane by Trans-Blot SD Semi-Dry Transfer Cell (Bio-rad). Following transfer, the membrane was blocked for 45 min in blocking buffer and then hybridized with the primary antibodies against p53, Bcl-2, CD44, c-Myc, cleaved caspase-3 and cleaved PARP at the appropriate dilution overnight at 4 °C. After washing three times with TBST buffer, the membrane was probed with peroxidase-conjugated goat anti-rabbit IgG as second antibody at 25 °C in the dark for 3 h. Protein bands were visualized by SuperSignal West Pico Chemiluminescent Substrate and quantified using densitometric analysis.

Plasmid construction of shRNA for down-regulating p53 expression. Two 64-nucleotide oligonucleotides, each containing the unique 19-nucleotide sequence and restriction enzyme cutting sites derived from the p53 transcript, were annealed (95 °C, 4 min; 95 °C to 25 °C, 1 h) and then ligated into the BamHI/HindIII restriction site of pSilencer 2.1-U6 neo plasmid (Ambion, TX, USA) by T4 DNA Ligase. The scrambled shRNA of p53 (scrambled control) were used as control for no target in the corresponding genome by shRNA-p53. Efficiency of RNA interference on p53 expression was estimated by semi-quantitative RT-PCR analyses (See supplemental materials).

Animal transplantation. For *in vivo* transformation, 2×10^7 EJ cells with shRNA-p53 or scrambled control and EJ cells without treatment were injected into the flank of athymic nude mice (4–6 weeks old, male, 18–25 g, each group, $n = 5$), respectively. Tumor growth was measured after 2 weeks from injection and at 5-day intervals for consecutive 4 weeks. After tumor formation, mice were treated with PRE (0 or 100 mg/kg body weight) through gavage (4 weeks, once daily). At the end of the experiment, mice were euthanized, and tumors were isolated and weighed. Tumor volume (V) was monitored by measuring the length (L) and width (W) with caliper and calculated according to the formula V [cm^3] = (length [cm]) × (width [cm]) × (width [cm]) / 2. Tumor tissues were retrieved for consecutive miR-34a expression analysis. All animal experiments were conducted in accordance with the guidelines regarding the care of experimental animals as approved by the Animal Research Central at Wuhan University.

Statistical analysis. All of the experiments were repeated at least three times. The data are expressed as the means ± standard deviation (SD) and analyzed using a *t*-test or one-way ANOVA to determine any significant differences. Differences were considered to be statistically significant if the corresponding *P* value was <0.05.

RESULTS

The qualitative analysis of total phenolic content and composition of pomegranate rind extract (PRE)

The total polyphenol contents of PRE were quantified by Folin–Ciocalteu method and expressed as gallic acid equivalents (GAE) in g/100 g plant material. The total polyphenol contents in samples were 27.58 ± 4.3%, derived from a standard curve of gallic acid. To explain the physiological effects due to polyphenols in PRE, we analyzed the polyphenol types of PRE. Peak distribution in the chromatographic profiles showed that punicalins, punicalagin A, punicalagin B and ellagic acid are the main absorption at 254 nm. The peak area ratio of these ellagitannins accounted for over 70% of the total peak area, and the retention time of each peak was calibrated to reference substance, respectively (see supplemental materials).

PRE suppressed EJ cell proliferation, but kept no antiproliferative effect on normal rat urinary bladder epithelial cells

In CCK assay, PRE attenuated cell viability with time (0–72 h) and dose (0–200 μg/mL) dependence (Fig. 1). The IC_{50} of PRE in EJ cells was 72 ± 4.1 μg/mL (48 h), and cell viability was sharply modified from 40 to 100 μg/mL of PRE. Moreover, RUBE cells showed insignificant sensitivity and less valuable IC_{50} (>200 μg/mL) to PRE treatment.

Caspase-dependent pathway was involved in PRE promoted apoptosis in EJ cells

Caspases are a group of intracellular proteases and activated by proteolytic cleavage, which is triggering the signaling of cells into apoptotic bodies during apoptosis. Generally, activated initiator caspases (including caspase -8 and -10) cleavingly activated a key downstream effector caspase-3, multiplying apoptotic signals in caspase-dependent pathway. To identify whether caspases are involved in PRE-induced apoptosis progress, different caspase inhibitors were added. Z-VAD-FMK (pan-caspase inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-DEVD-FMK (caspase-3 inhibitor) obviously reversed the molecular death by 0.7-, 0.8- and 0.5-fold, respectively, but Z-AEVD-FMK (caspase-10) induced only increased 8% of viable cells, compared to PRE sole treatment

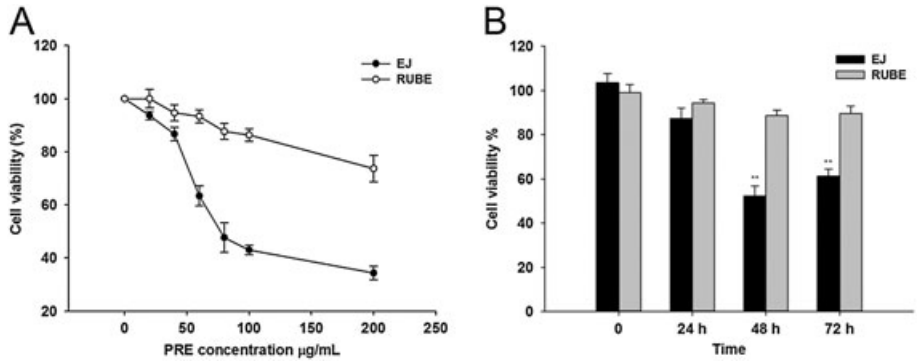

Figure 1. Anti-proliferative effects of PRE on human bladder cancer EJ cells and normal rat urinary bladder epithelial cells. (A) Human bladder cancer EJ cells and normal rat urinary bladder epithelial cells were treated with 0–200 μg/mL for 48 h. Cell viability was determined by CCK assay and normalized to vehicle-treated controls. (B) Two kinds of cells were subjected with 100 μg GAE/mL for 24, 48 and 72 h, respectively. Cell viability was determined by CCK assay as described in Methods. The values given are the mean ± SD of five independent experiments. $*P < 0.05$ compared to mock control in (B).

(Fig. 2**A)**. In semi-quantitative RT-PCR assay, caspase-3 mRNA was increased to nearly 1.8-fold of control in 70 μg/mL of PRE group. Western blot assay was performed to evaluate the levels of cleavage caspase-3 and PARP in PRE-induced apoptosis. As shown in **Fig.** 2**B** and 2**C**, after 48-h PRE treatment, protein expression of cleavage caspase-3 was suppressed in PRE with z-DEVD-FMK treated cells by 0.6-fold compared with single dose 100 μg/mL of treated cells. Moreover, the protein expression of both cleavage caspase-3 and PARP was dose-dependently enhanced in PRE treated groups by 1.5-, 2.2- and 2.5-fold and 1-, 1.7- and 1.6-fold, respectively (**Fig.** 2**D–E**).

PRE regulated c-Jun and p53 protein expression and miR-34a expression was mediated by p53 in PRE-induced apoptosis

In our findings, the protein expression of p53 was significantly increased by 1.7-, 1.6- and 1.8- fold, and c-Jun protein was decreased by 0.6-, 0.3- and 0.2-fold in

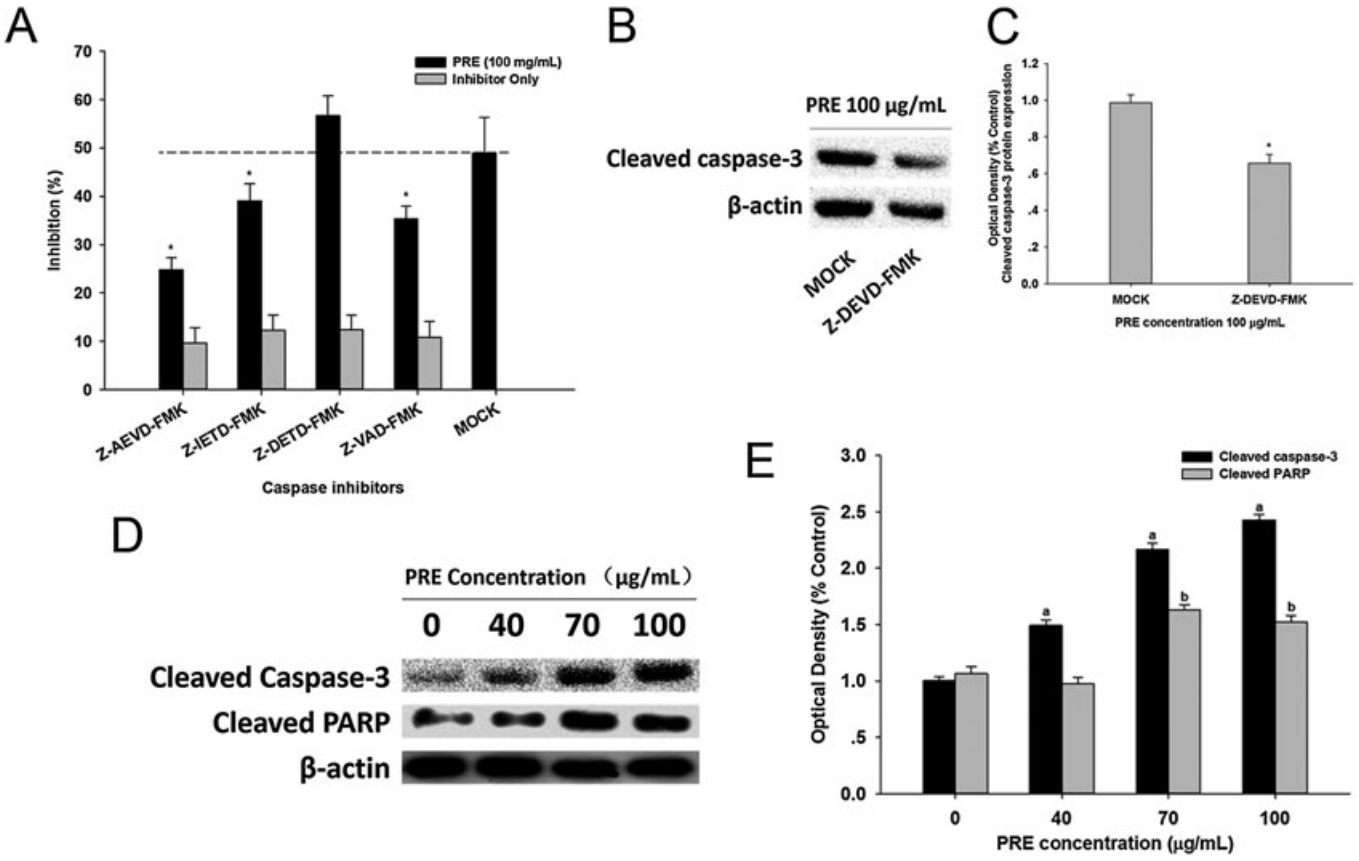

Figure 2. Caspase-dependent signaling involved in PRE-induced EJ cell death. (A) Effects of caspase inhibitors on 70 μg/mL PRE-induced EJ cell death. The cells were cultured in the absence or presence of Z-VAD-FMK (20 μmol/L), Z-DEVD-FMK (20 μmol/L), Z-IETD-FMK (20 μmol/L) and Z-AEVD-FMK (10 μmol/L), 2 h prior to the addition of PRE, and then incubated for 48 h. (B) Western blot analysis for the expression of cleaved caspase-3 after 48 h of administration of 100 μg/mL PRE in the absence or presence of Z-DEVD-FMK. (C) Western blot analysis for the expression of cleaved caspase-3 and PARP after 48 h of administration in different concentration. (D, E) Bars represent the intensity of the bands of (B) and (D) quantitated by densitometry, respectively. $n = 5$. Mean ± SD. $*p < 0.05$ vs 70 μg/mL PRE group.

treatment with 40, 70 and 100 μg/mL of PRE compared to the vehicle-treated control, respectively (Fig. 3E). Likewise, Bcl-2, c-Myc and CD44 mRNA and protein expression were concomitantly suppressed during the PRE-induced p53/miR-34a axis inversion (Fig. 3D, E). The miR-34a expression was associatively elevated by approximately 1.75- and 1.7- fold with gradually elevated p53 protein level in 70 and 100 μg/mL of PRE, respectively (Fig. 3C).

The miR-34a selectively suppressed the c-Myc and CD44, but wasn't intermediate in Bcl-2 expression

Next, we tendentiously investigated whether the decrease of these genes was contributed to enhanced miR-34a expression by PRE-induced p53 activation. To this purpose, antagomiR of miR-34a was transfected and incubated for 2 h in EJ cells before PRE-administration. The results showed that antagonistic miR-34a brought increase of c-Myc and CD44 protein expression by 1.8- and 1.4-fold respectively (Fig. 4B–C). However, in this study the Bcl-2 protein showed no significant difference in miR-34a alteration, which indicated that Bcl-2 probably wasn't a direct target of PRE-induced miR-34a transcription (Fig. 4B–C).

PRE inhibits the growth of EJ xenografts in Balb C nude mice

We measured the effects of PRE on growth of EJ xenografts. PRE inhibited EJ tumor growth (volume) in Balb C nude mice (Fig. 5A). PRE had no effect on the body weight of EJ tumor bearing mice. We have not observed any toxicity in the liver, spleen and intestine of mice treated with PRE, suggesting that it is a safe natural product. Moreover, we also isolated the micro-RNAs in xenografts and found that miR-34a were up-regulated in PRE-treated mice. However, shRNA-p53 group show larger tumor size and lower miR-34a expression level than PRE-treated group (Fig. 5B).

DISCUSSION

Generally, abundant ellagic acid, ellagitannins and a handful of flavonoids are considered as the essential antioxidant ingredients in pomegranate fruit. In the present study, we assessed main polyphenolic components of PRE by HPLC-MS, and the results indicated that ellagitannins named punicalins, punicalagin A, punicalagin B and ellagic acid were the main composition

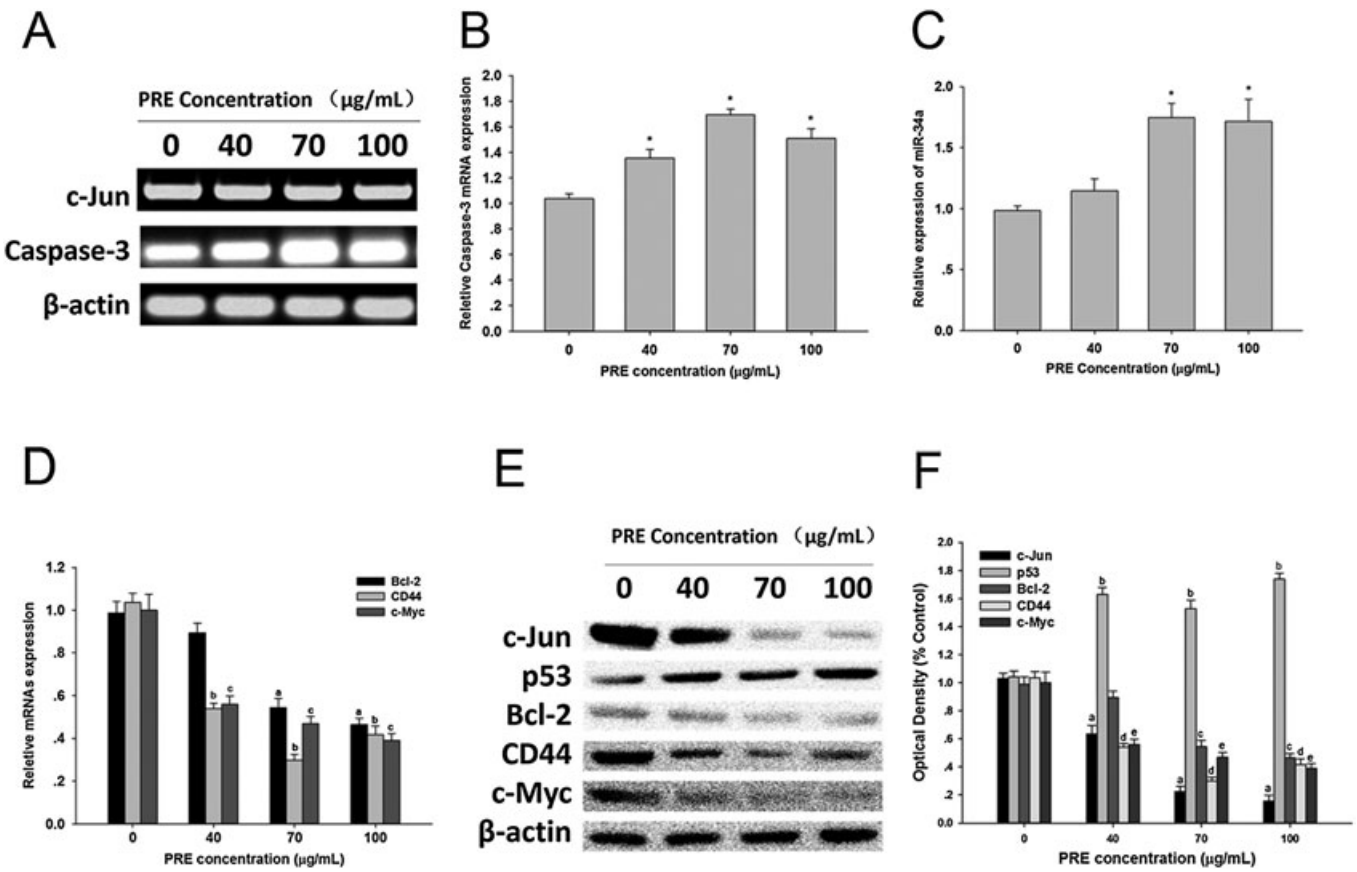

Figure 3. The effects of PRE on p53 and miR-34a expression in the process of cell apoptosis. (A) The mRNA and c-Jun expression of caspase-3 after 48 h of administration of 100 μg/mL PRE was assessed by semiquantitative PCR. (B) Bars represent the intensity of the caspase-3 bands of in (A), quantitated by densitometry. PRE regulated the expression of (C) miR-34a, as well as (D) mRNA and (E) protein of c-Jun, p53, Bcl-2, CD44 and c-Myc in EJ cells. (F) Bars represent the intensity of the bands of (E), quantitated by densitometry. Cells were treated with vehicle (control) or different concentration of PRE (40, 70 and 100 μg/mL) for 48 h. All experiments were performed as described in Methods and were performed at least three times, and results were expressed as mean ± standard error. *Indicates significant changes at $P < 0.05$. PCR as described under Methods. Bars marked a, b, c, d and e indicated significant difference compared to each vehicle control, respectively.

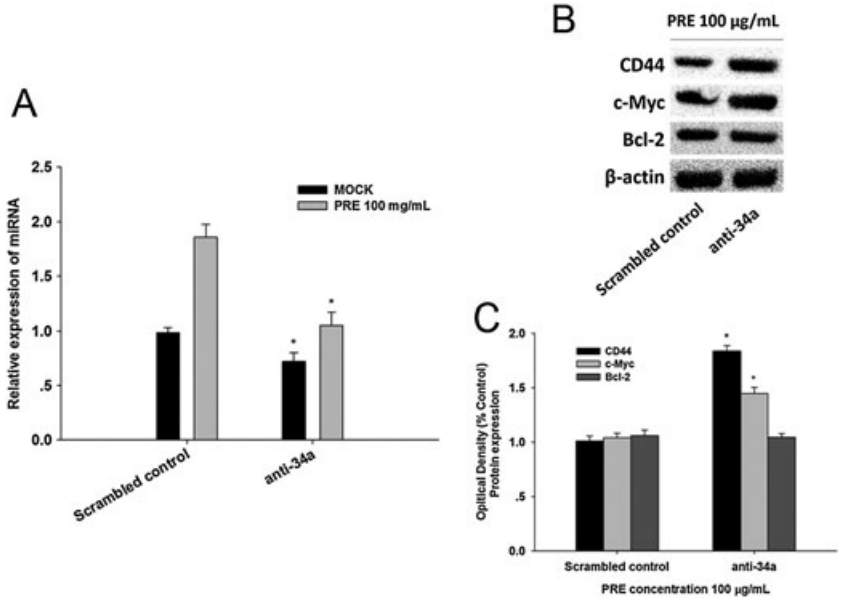

Figure 4. Effects of PRE on miR-34a levels and its potential targets in EJ cells transfected with antagomiR of miR-34a (anti-34a). Cells were transfected with 20 nM of anti-34a and subsequently subjected to 100 μg/mL PRE. (A) PRE elevated the miR-126 suppressed by anti-34a. (B) The decreased expression of miR-126 was accompanied by a decreased expression of CD44 and c-Myc, but not Bcl-2. (C) Bars represent the intensity of the bands of (B), quantitated by densitometry. All experiments were performed as described in methods $n = 5$, and results were expressed as mean ± standard error. *Indicates significant changes at $P < 0.05$.

of PRE. Then it was found that PRE could selectively suppress the viability of human bladder cancer cells ($IC_{50} = 70$ μg/mL) compared to PRE-insensitive RUBE cells ($IC_{50} > 200$ μg/mL).

The caspase-dependent pathway was vulnerable activated by plant polyphenols during apoptosis (Kothakota *et al.,* 1997). We firstly employed pan-caspase inhibitor z-VAD-FMK to assess whether caspases worked in EJ cell viability alteration by PRE treatment and found that the pan-caspase function in this process was positive. Then, specific inhibitors against caspase-3, -8 and -10 generation were administrated to study contributory caspases to the progress, and only caspase-10 was assessed to have no effect during EJ cell apoptosis. In order to further confirm the characteristic cell apoptosis mediated by caspase-3, the protein expressions of cleavage caspase-3 and PARP were examined, and the levels of the two proteins also increased during EJ cell apoptosis.

A novel molecular mechanism of miR-34a targeted by polyphenols was also discussed, and this achievement was involved in p53 activation. Several tumor suppressor functions of p53 are regulated by p53-induced miRNA transcription (Hermeking, 2007). The subtypes of miR-34 family have been identified as the most prevalent p53-mediated miRNAs. Generally, the miR-34a located at 1p36 locus is frequently expurgated in carcinoma (Welch *et al.,* 2007). The miR-34a is also commonly inhibited in tumors compared with peritumorial tissues of clinical cancerous samples (Hermeking, 2012). It was found that p53 protein can specifically activate the miR-34a transcription by binding to responsive elements of miR-34a (Raver-Shapira *et al.,* 2007). Proliferation (Fujita *et al.,* 2008), epithelial to mesenchymal transition (Hahn *et al.,* 2013), invasion (Garofalo *et al.,* 2013) and metastasis (Kaller *et al.,* 2011) of cancer cells are commonly misadjusted in abnormal loss of

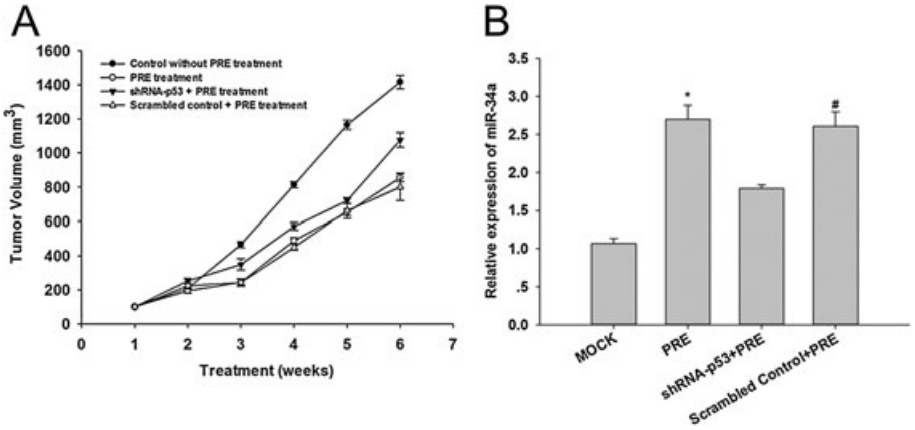

Figure 5. Effects of PRE with/without shRNA-p53 on EJ xenografts and miR-34a expression in Balb C nude mice. (A) EJ cells (2 × 106 cells mixed with Matrigel, 50:50 ratio) were subcutaneously implanted into the flanks of Balb C nude mice. Tumor bearing mice were treated with PRE (0 or 100 mg/kg body weight) through gavage (Monday through Sunday, once daily) for 4 weeks. (B) miR-34a expression at the end of gavage. Mice were euthanized, and tumors were obtained for further use. The values given are the mean ± SD of five independent experiments. *$P < 0.05$ compared to mock control. #$P < 0.05$ compared to shRNA-p53 group.

miR-34a. The p53/miR-34 axis is distinguished as a crucial target of cancer prevention, and induction of miR-34 expression by artificial and drug strategy may be momentous for future cancer therapeutics. A previous report demonstrated that low expression of miR-34a was relevant to the malignancy and tumor size of bladder cancer in patients (Li *et al.,* 2014). Likewise, miR-34a was involved in suppressing excessive proliferative activity of T24 human bladder cancer cell (Wang *et al.,* 2013). Moreover, c-Jun was also a negative regulator of p53 which is involved in tumor invasion and metastasis (Schreiber *et al.,* 1999). In our study, abnormal p53 expression was reversed as PRE exposure, accompanied by the increase of miR-34a expression. What's more, the effect of PRE on mir-34a re-expression in EJ bladder cancer cells was blocked by shRNA-p53 mediated p53 silence. To our delight *in vivo* analysis revealed a prominently similar trend as our *in vitro* data, which confirmed that miR-34a inhibits cancer proliferation through induction of p53 activation in a time-depen dent manner. Moreover, since PRE is a nontoxic compound, it may be securely applied for the treatment and/or prevention of bladder cancer. Though the c-Jun protein expression was suppressed by PRE, the c-Jun mRNA expression showed no statistical significance among groups (Fig. 3). We suppose that c-Jun protein expression probably tends to be modified by PRE induced ubiquitylation, and deep going study is necessary to verify this hypothesis.

Gain of chromosome 8q has been identified in the process of bladder cancer formation (Moore *et al.,* 2002). The c-Myc is a kind of oncogenes regulating cell proliferation and differentiation and at 8q24 chromosomal loci (Grisanzio and Freedman, 2010). Overexpression of c-Myc was related to deteriorative progress of bladder cancer, and its expression level had been a marker in grading of tumors (He *et al.,* 1998). CD44 is a bunch of adhesion molecules encoded by a single gene with Ras-MEK-ERK pathway mediated alternative splicing, which is classified as the marker on cancer stem cell surface (Deschoolmeester *et al.,* 2010). In a previous study, CD44 has also been characterized in physiological processes as well as cancer cell invasion and metastasis (Orian-Rousseau, 2010). Our results indicated that in the process of EJ cell apoptosis, c-Myc and CD44 was the direct targets of miR-34a in PRE-treated EJ cells, and the modification on c-Myc and CD44 protein expression could be blocked by miR-34a antagonist.

According to the results of this study, it is clearly indicated that polyphenols in pomegranate rind extract have an anti-proliferative effect and promote apoptosis in human bladder cancer cells, but it's less insensitive to the cell viability of normal rat urinary bladder epithelial cells. The pro-apoptotic activity contributes to activation of caspase dependent pathway from caspase-8 to -3, rather than -10. In the process of cell apoptosis and necrosis, c-Jun protein expression diminished, combined with p53 protein accumulation. Such switch of c-Jun and p53 expressing pattern by PRE enhanced the expression of miR-34a, the effector of p53 downstream target. Finally, in the investigation of potential target molecules of miR-34a, c-Myc and CD44 appeared due to the down-regulation of the mRNA and protein expression. Though *in vitro* and *in vivo* evaluation was warrant for the anticarcinogenic function of PRE, further *in vivo* estimation is still necessary to study potential biological toxicity and therapeutic effects of pomegranate rind extracts. Due to the similarity of polyphenol constituents in pomegranate edible parts and rind, we are optimistic to the safety of pomegranate rind polyphenols if it was subjected in clinical assessment.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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