Available online at www.sciencedirect.com

ScienceDirect

www.nrjournal.com

Original Research

Punicalagin from pomegranate promotes human papillary thyroid carcinoma BCPAP cell death by triggering ATM-mediated DNA damage response

Xin Yao[a,b,1], *Xian Cheng*[a,1], *Li Zhang*[a,c], *Huixin Yu*[a], *Jiandong Bao*[a], *Haixia Guan*[d], *Rongrong Lu*[b,*]

[a] *Key Laboratory of Nuclear Medicine, Ministry of Health, Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi, Jiangsu, China*

[b] *School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China*

[c] *State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu, China*

[d] *Department of Endocrinology and Metabolism and Institute of Endocrinology, the First Hospital of China Medical University, Shenyang, Liaoning, China*

ARTICLE INFO

Article history:
Received 4 May 2017
Revised 4 September 2017
Accepted 5 September 2017

Keywords:
Punicalagin
Papillary thyroid carcinoma
BCPAP cell
DNA damage response
Ataxia-telangiectasia mutated gene-encoded protein

ABSTRACT

Punicalagin (PUN), a component derived from pomegranate, is well known for its anticancer activity. Our previous work revealed that PUN induces autophagic cell death in papillary thyroid carcinoma cells. We hypothesized that PUN triggers DNA damage associated with cell death because DNA damage was reported as an inducer of autophagy. Our results showed that PUN treatment caused DNA breaks as evidenced by the significant enhancement in the phosphorylation of H2A.X. However, reactive oxygen species and DNA conformational alteration, 2 common inducing factors in DNA damage, were not involved in PUN-induced DNA damage. The phosphorylation of ataxia-telangiectasia mutated gene-encoded protein (ATM) but not ataxia telangiectasia and Rad3-related protein (ATR) was up-regulated in a time- and dosage-dependent manner after PUN treatment. KU-55933, an inhibitor of ATM, inhibited the phosphorylation of ATM induced by PUN and reversed the decreased cell viability caused by PUN. Thus, we demonstrated that PUN induces cell death of papillary thyroid carcinoma cells by triggering ATM-mediated DNA damage response, which provided novel mechanisms and potential targets for the better understanding of the anticancer actions of PUN.

Abbreviations: ATM, ataxia-telangiectasia mutated gene-encoded protein; ATR, ataxia telangiectasia and Rad3-related protein; PBS, phosphate-buffered saline; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DDR, DNA damage response; DSBs, DNA double-strand breaks; H2A, histone 2A; PTC, papillary thyroid carcinoma; PUN, punicalagin; ROS, reactive oxygen species; SRB, sulforhodamine B; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

* *Corresponding author at:* School of Food Science and Technology, Jiangnan University, 1800 Lihu Ave, Wuxi, Jiangsu 214122, China. Tel.: +86 510 85329061; fax: +86 510 85912155.

E-mail addresses: yaoxin150@163.com (X. Yao), chengxian@jsinm.org (X. Cheng), zhangli@jsinm.org (L. Zhang), yuhuixin@jsinm.org (H. Yu), baojiandong@jsinm.org (J. Bao), hxguan@vip.126.com (H. Guan), lurr@jiangnan.edu.cn (R. Lu).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.nutres.2017.09.001
0271-5317/

1. Introduction

Polyphenols, a large class of chemical compounds, are abundant in our daily diet, especially in fruits and drinks, such as apple, pomegranate, tea, and red wine. Owing to their antioxidant, antimicrobial, and anticancer properties, polyphenols have aroused attentions from many researchers in related fields [1]. Punicalagin (PUN), derived from pomegranate peel or seeds, is the largest known polyphenol for its molecular weight reaches 1084.71 g/mol. Punicalagin possesses great anticancer efficacy. It has been reported that PUN exerts inhibitory effect on prostate cancer [2], lung cancer [3], breast cancer, and cervical cancer [4]. However, its pharmacological mechanism in thyroid carcinoma has not been fully defined.

Thyroid carcinoma belongs to endocrine malignancies and is classified into 4 types including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, anaplastic thyroid carcinoma, and medullary thyroid carcinoma, among which PTC is the most common clinical type. Thyroid carcinoma has a high incidence worldwide in recent years [5,6]. Most patients have a good prognosis after the traditional therapies targeting thyroid carcinoma including surgery, chemotherapy, and radiotherapy. However, there are still a number of patients who are not responsive to radiotherapy and need a more personalized treatment [7]. Therefore, we are focused on seeking new therapies or new drugs for thyroid carcinoma treatment.

DNA damage can be triggered by several stimuli such as oxidative stress, ionizing radiation, and mutagenic chemicals, and one of the most common DNA lesions is DNA double-strand breaks (DSBs), which usually results in DNA damage response (DDR). Once DDR was initiated by the recognition of specialized proteins, several signaling cascades are involved in the process of damage repairing. Homologous recombination and nonhomologous end joining coordinately participate in the repairing of DSBs [8], and ataxia-telangiectasia mutated gene-encoded protein (ATM) plays a central role in DDR mainly through initiating homologous recombination [9]. DNA damage response is implicated in many physiological and pathological processes [9], and the expression of DSBs repairing genes is usually up-regulated in cancers [10], which may provide prospective targets for tumor therapy.

DNA damage will lead to DNA repair, a cell-cycle checkpoint, and the outcome can be cell apoptosis. Autophagy has also been reported to be involved in DDR. However, the underlined mechanisms of DNA damage-induced autophagy remained unclear [11]. DNA damaging agents can induce autophagy including the activation of mammalian target of rapamycin complex 1 repressor, which reverses the inhibitory effect of mammalian target of rapamycin complex 1 on autophagy [12]. Our latest research has shown that PUN induces autophagic cell death of BCPAP [13]. This result directed our research to determine if the DDR is involved in the process of PUN-induced cell death. In the present study, we detected p-H2A.X level, performed terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, and analyzed DNA conformation, reactive oxygen species (ROS) content, and the phosphorylation of ATM and ataxia telangiectasia and Rad3-related protein (ATR) to further define the pharmacological mechanism of PUN in papillary thyroid cancer cells. We found that PUN triggered DDR through an ATM-dependent manner in PTC BCPAP cells, which further promoted cancer cell death. Our results advance the medical value of PUN and provide more evidence for its potential application in PTC therapy.

2. Methods and materials

2.1. Chemicals, reagents, and antibodies

Punicalagin (high-performance liquid chromatography ≥98%) was purchased from klamar-reagent (Shanghai puzhen Biotechnology Co, Ltd, Shanghai, China). Albumin from bovine serum, methyl thiazolyl tetrazolium (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sangon (Shanghai, China). Newborn calf serum was purchased from Zhejiang Tianhang Biological Technology Co, Ltd (Zhejiang, China). Sulforhodamine B (SRB) and one Step TUNEL Apoptosis Assay Kit were purchased from Beyotime Biotechnology (Shanghai, China). 4′,6-Diamidino-2-phenylindole and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (MO, USA). Cisplatin was purchased from Jiangsu Hansoh Pharma Group Co, Ltd (Jiangsu, China). KU-55933 was purchased from Abcam (Cambridge, UK). The antibodies used in this article were as follows: anti-ATM (sc-23 921), anti-ATR (sc-28 901), and anti–β-actin (sc-47 778) were purchased from Santa Cruz Biotechnology (CA, USA). Anti–phospho-histone H2A.X (Ser139, cst, no. 9718P), anti–phospho-ATM (Ser1981, cst, no. 5883P), and anti–phospho-ATR (Ser428, cst, no. 2853P) were purchased from Cell Signaling Technology (MA, USA).

2.2. Cell culture and treatments

PTC BCPAP cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were incubated with 1640 culture medium (Gibco, Grand Island, NY, USA), which contains 100 U mL^{-1} penicillin (CHINO Pharmaceutical Co, Ltd, Shijiazhuang, China), 100 mg L^{-1} streptomycin (Shandong Lukang Pharmaceutical Co, Ltd, Shandong, China), and 10% newborn calf serum (Sijiqing, Hangzhou, China). The cells were placed at a constant temperature incubator (Thermo Electron Corporation, Waltham, MA, USA) at the condition of 37°C, 5% CO_2, saturated humidity. Punicalagin was dissolved in methanol to a concentration of 50 mM and stored at –20°C. Unless otherwise specified, BCPAP cells (passage 3) were treated with PUN at different concentrations (25, 50, and 100 μM) for 24 h. The solvent control contained equal amount of methanol to that of 100 μM PUN. KU-55933 was dissolved in DMSO to a concentration of 25 mM as a stock solution.

2.3. SRB assay

Sulforhodamine B assay was performed to test the cytotoxicity of the drug [14] and some proper modifications were made. Cells (passage 3) grown to logarithmic phase were seeded in 96-well plate (Costar; Corning Incorporated, Corning, NY, USA). After treatment, the supernatant was removed and 200 μL 10% trichloroacetic acid was added. Then the cells

were fixed at room temperature for 5 minutes and 4°C for 1 hour. Next, the supernatant was discarded and the cells were washed by phosphate-buffered saline (PBS) buffer (0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g Nacl, 1.94 g $Na_2HPO_4 \cdot 12H_2O$/L) 3 times and dried in the air. The cells were stained with 100 μL 0.4% SRB dye liquor (appropriate SRB dissolved in 1% glacial acetic acid) at 37°C for 30 minutes and then washed by 1% glacial acetic acid until the free dye liquor was absolutely washed out. Then the cells were dried in the air, and after that, 150 μL 10 mM Tris was added. Next, the 96-well plate was placed on a shaker for 30 seconds to dissolve the precipitates adequately. Finally, the absorbance at 565 nm was determined by a microplate reader (μQuant, BioTek, VT, USA).

2.4. *Western blot*

Western blot was performed as we described before [13], and minor modifications had been made. After treatment with PUN, the cells (passage 3) were collected and washed by ice-cold PBS. Cell deposits were resuspended with ice-cold cell lysis (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 2 mM ethylenediaminetetraacetic acid, 1% [wt/vol] Nonidet P-40, 0.02% [wt/vol] sodium azide) containing 1% protease inhibitors (phenylmethylsul-fonyl fluoride), and all samples were placed at –80°C to room temperature for freezing and thawing, which was repeated 3 times. Then the samples were centrifuged at 12 000 rpm, 4°C for 15 minutes, and the supernatant was collected. Protein concentration was

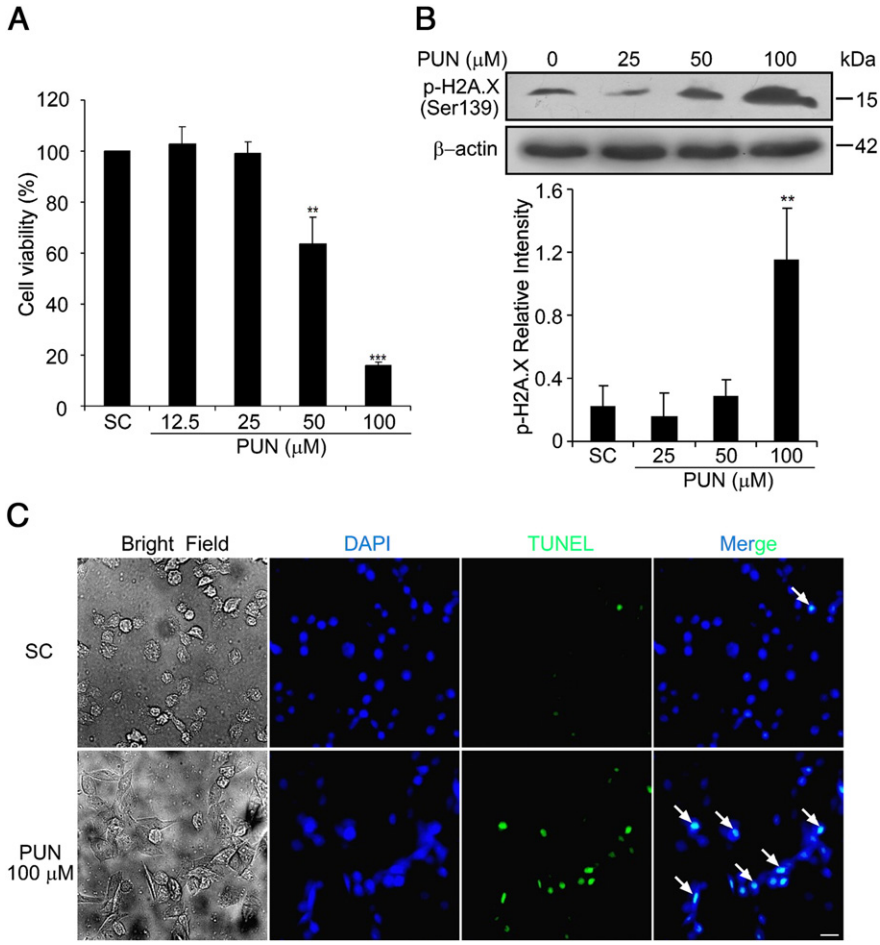

Fig. 1 – Punicalagin inhibits the cell viability and induces DNA damage in BCPAP cells. A, Punicalagin inhibits the cell viability of BCPAP cells. BCPAP cells were seeded in 96-well plate and treated with indicated concentrations of PUN for 24 hours, respectively. Another group was set as a solvent control in which BCPAP cells were treated with equal amount of methanol to that of 100 μM PUN for 24 hours. The cell viability was determined by SRB assay. Each value stands for mean ± SD of 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by *t* test. **P < .01, ***P < .001 vs solvent control. B, Punicalagin enhances the phosphorylation of H2A.X BCPAP cells. Cells were harvested after being incubated with PUN at various concentrations for 24 hours, and the phosphorylation of H2A.X was determined by Western blot. β-Actin was used as a control. Quantitative result was shown as histogram, and the values were presented as means ± SD of 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by *t* test. **P < .01 vs solvent control. C, Punicalagin induces DNA breaks in BCPAP cells. The BCPAP cells were treated with or without 100 μM PUN for 24 hours. The representative images (color figures) for the TUNEL staining were shown. The scale bar represents 20 μm.

determined by BCA or G250 Coomassie brilliant blue method. Next, equivalent protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred on nitrocellulose filter membrane. After being stained by Ponceau S dye liquor, the membrane was blocked by 5% nonfat milk for 1 hour and then incubated with primary antibody at 4°C overnight. Next, it was washed 3 times by Tris-buffered saline with Tween-20 buffer (137 mM NaCl, 20 mM Tris-HCl, 0.1% Tween-20 [pH 7.6]) and incubated with secondary antibody for another 1 hour and then washed 3 times again. Finally, the target protein band was visualized by enhanced chemiluminescence, followed by the exposure on X-OMAT BT film (Eastman Kodak, NY, USA).

2.5. *TUNEL assay*

TUNEL assay was conducted as we described in the previous study [15], and some proper adjustments had been made. BCPAP cells (passage 3) were incubated on a coverslip overnight and then treated with PUN for 24 hours. After being washed with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 30 to 60 minutes and then washed again. Next, 0.1% ice-cold Triton X-100 was used to penetrate the cell membrane for 2 minutes. After being washed with PBS for twice, the cells were incubated with TUNEL solution, a mixture made according to the manufacturer's instructions, at 37°C away from light for 60 minutes and then washed with PBS 3 times. Finally, the cells were stained with 4′,6-diamidino-2-phenylindole (1 μg/mL) for 5 minutes and observed by a fluorescence microscope (IX81; Olympus, Tokyo, Japan).

2.6. *DNA conformational change assay*

As we described in the previous work [16], the plasmid pcDNA 3.1 (+) was incubated with different concentrations of PUN (0-100 μM) at 37°C for 12 hours and the DNA was separated by 1% agarose gel electrophoresis. Cisplatin (0-100 μM) was used as a positive control.

2.7. *ROS production measurement*

Production of ROS was measured by DCFH-DA assay [15]. 2′,7′-Dichlorofluorescin diacetate is able to penetrate cell membrane freely and be resident in cells through hydrolyzing into DCFH, which does not contain fluorescence. However, intracellular ROS oxidizes DCFH into DCF, which is a form of fluorescence. Thus, the intensity of fluorescence reflects the activity of ROS.

The BCPAP cells (passage 3) were treated with PUN for 24 hours and collected with trypsin. After centrifuge for 5 minutes, the precipitates were stained with 10 μM DCFH-DA at 37°C away from light for 30 minutes. Next, the cells were washed and then resuspended in fresh PBS. All samples were detected by flow cytometry at FL1-H channel. The results were analyzed by Cell Quest (Becton, Dickinson and Company, NJ, USA) and FlowJo software (Stanford University, CA, USA).

2.8. *MTT assay*

Methyl thiazolyl tetrazolium assay was conducted as previous work [13]. The logarithmic-phase cells (passage 3) were seeded in 96-well plate (Costar; Corning Incorporated). After drug treatment, 40 μL MTT (2 mg/mL) was added and the cells were incubated at 37°C for 4 hours. The supernatant was

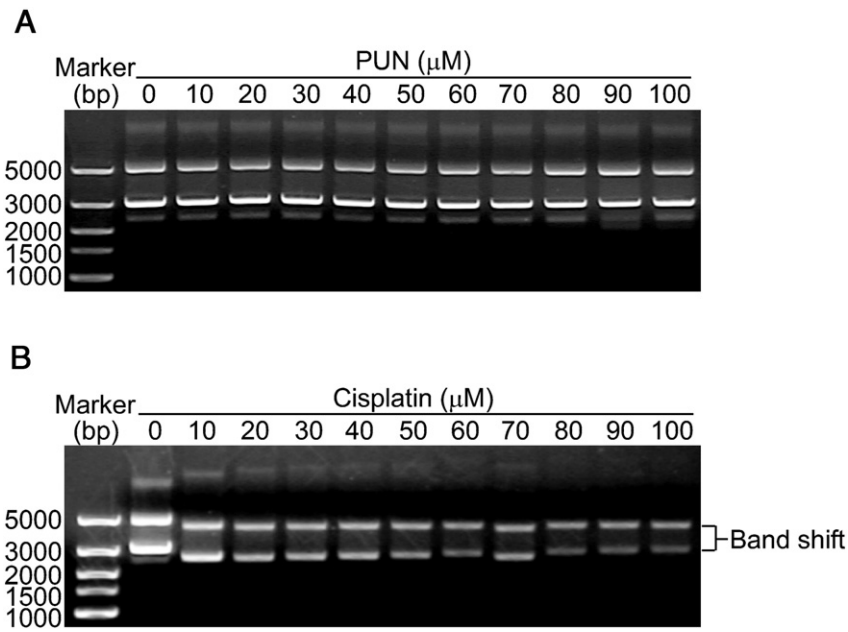

Fig. 2 – Punicalagin fails to change the DNA conformation. A, The results show that pcDNA 3.1 (+) plasmid (500 ng in each sample) was incubated with different concentrations of PUN (0–100 μM) at 37°C for 12 hours, and all samples were detected by 1% agarose gel electrophoresis. B, The results show that pcDNA 3.1 (+) plasmid (500 ng in each sample) was treated by cisplatin (0-100 μM) as described in panel A, which was served as a positive control.

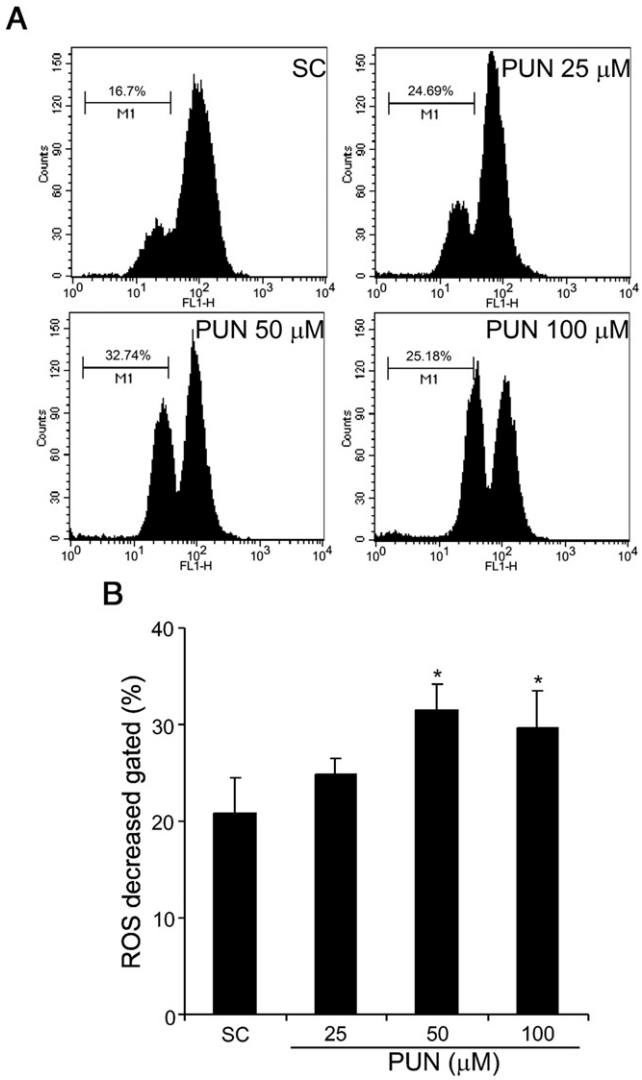

Fig. 3 – Punicalagin-induced DNA damage is independent of ROS. A, The results show that BCPAP cells were treated with PUN at different concentrations for 24 hours and then stained with 10 μM DCFH-DA at 37°C away from light for 30 minutes. All samples were analyzed by flow cytometry at FL1-H channel, and the data in panel A stand for the percent of ROS-decreased gated cells from one test. B, The histogram was representative of the percent of cells with ROS decrease, and the values were presented as means ± SD from 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by t test. *P < .05 vs solvent control.

removed and 100 μL DMSO was added. Then the 96-well plate was placed on a shaker for 5 minutes to dissolve the precipitates adequately. Finally, the absorbance at 490 nm was determined by a microplate reader (μQuant).

2.9. Statistical analyses

All data are shown as means ± SD of 3 independent experiments, the statistical significance was determined by 1-way analysis of variance (ANOVA), and the comparison between 2 groups was made by a t test [13]. Differences between mean values were determined as follows: *P < .05 represents significant difference; **P < .01, highly significant difference; and ***P < .001, highly significant difference.

3. Results

3.1. PUN inhibits the cell viability and induces DNA damage in BCPAP cells

We first examined the growth-inhibitory effect of PUN on BCPAP cells by SRB assay. Punicalagin treatment at 12.5 and 25 μM did not exert significant inhibitory effect on BCPAP cell viability compared with the solvent control group. However, with higher dosages, PUN at 50 and 100 μM caused the cell viability of BCPAP cells to decline to 63.46% ± 10.57% and 16.10% ± 1.16%, respectively (Fig. 1A), which is in accordance with our previous work [13].

Histone 2A (H2A) plays a vital role in the maintenance of chromatin [17]. Its various variants such as Macro-H2A, H2A.X, and H2A.Z differ in the capacity binding to nucleosome and DNA and participate in various events [18]. H2A.X is an important DNA damage sensor, which can be found in almost all organisms, and it is phosphorylated quickly once DNA breaks occur [19]. After treatment with PUN, the phosphorylation of H2A.X was concentration-dependently up-regulated. The increase of p-H2A.X in the 100-μM group was significantly up-regulated to 5-fold compared with the solvent control group ($P < .01$; Fig. 1B). In addition, the DNA breaks were also determined by TUNEL assay. When genomic DNA breaks appear in the nucleus, the naked 3′-OH can be targeted by the fluorescein-dUTP probe under the catalysis of terminal deoxynucleotidyl transferase and finally detected as green fluorescence spots, namely, TUNEL-positive cells. As shown in Fig. 1C, 100 μM PUN treatment increased the number of TUNEL-positive cells compared with solvent control group. Taken together, our results demonstrated that PUN induces DNA damage in BCPAP cells.

3.2. PUN fails to change the DNA conformation

In some cases, DNA conformational change, which may be triggered by mental ions, radiation, or chemotherapy drugs (such as cisplatin) [20], is involved in the process of DNA damage. Therefore, we probed the effects of PUN on DNA conformation. As shown in Fig. 2A, the DNA plasmids incubated with various dosages of PUN varied from 10 to 100 μM showed no band shift compared with the nontreated group. To get further confirmation, we compared the effects of PUN with that of cisplatin, which serves as a positive control. As shown in Fig. 2B, obvious band shifts were detected in the positive control groups treated with cisplatin from 10 to 100 μM. It is reported that cisplatin changes DNA conformation through binding with DNA and further unwinding negative supercoiling then inducing positive supercoiling [21]. Therefore, PUN induces DNA damage in BCPAP cells without affecting the DNA conformation.

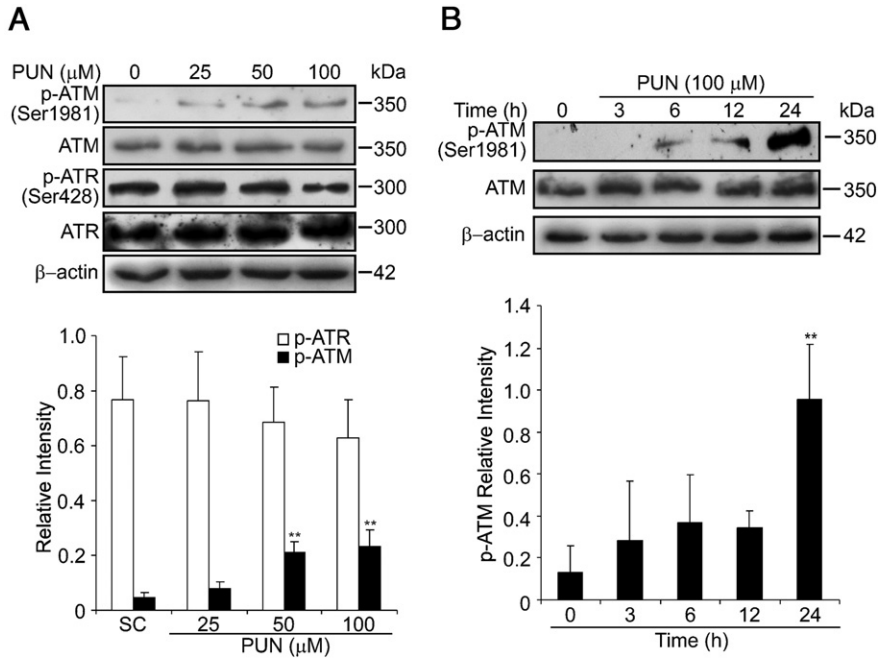

Fig. 4 – ATM, but not ATR, is involved in PUN-induced DDR in BCPAP cells. A, Punicalagin augments the phosphorylation of ATM in a dose-dependent manner but has no effect on the phosphorylation of ATR. BCPAP cells were collected after treatment with PUN at different doses for 24 hours. The protein levels of ATM, p-ATM, ATR, and p-ATR were detected by Western blot. β-Actin was used as a control. Quantitative analysis was shown in the bottom histogram, and the values were presented as means ± SD of 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by t test. **P < .01 vs solvent control. B, Punicalagin increases the phosphorylation of ATM in a time-dependent manner. BCPAP cells were harvested after treatment with 100 μM of PUN for indicated times, and ATM and p-ATM levels were detected by Western blot. β-Actin was used as a control. Quantitative analysis was shown in the bottom histogram, and the values were presented as means ± SD of 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by t test. **P < .01 vs solvent control.

3.3. *PUN-induced DNA damage is independent of ROS*

Reactive oxygen species, as a produced endogenous stimulus, is a common inducer triggering oxidative DNA damage [22]. A number of chemicals, such as fluconazole, can cause oxidative DNA damage through ROS production [23]. After treatment with PUN in various concentrations, the intracellular level of ROS in BCPAP cells did not increase but slightly decrease, especially in the 50- and 100-μM group with 31.45% ± 2.74% and 29.59% ± 3.84% ROS-decreased gated

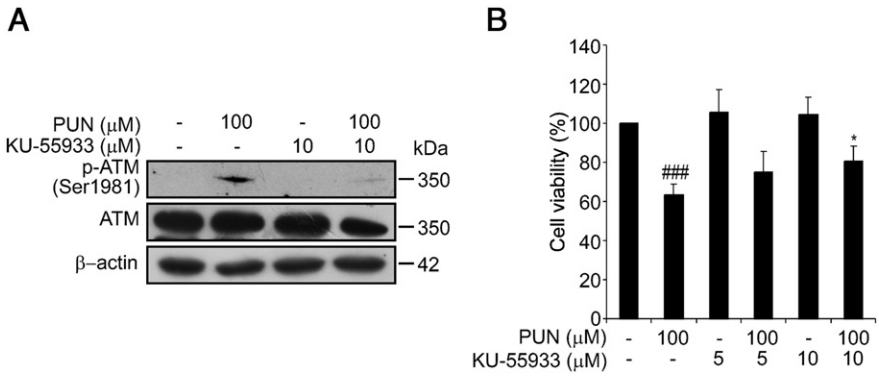

Fig. 5 – ATM-mediated DDR is involved in PUN-induced cell death of BCPAP cells. A, BCPAP cells were treated with 100 μM of PUN in the presence or absence of KU-55933 (10 μM) for 24 hours, and p-ATM and ATM levels were detected by western blot. β-Actin was used as a control. B, BCPAP cells were treated with 100 μM of PUN in the presence or absence of KU-55933 (5 and 10 μM) for 24 hours, and the cell viability was measured by MTT assay. Each value represents means ± SD of 3 independent experiments. The statistical significance was determined by 1-way ANOVA, and the comparison between 2 groups was made by t test. ###P < .001 vs solvent control; *P < .05 vs PUN treated-alone group.

cells, respectively ($P < .05$; Fig. 3). These data suggested that ROS is not involved in the PUN-induced DNA damage.

3.4. *ATM, but not ATR, is involved in PUN-induced DDR in BCPAP cells*

ATM and ATR are 2 important molecules involved in DDR, because they are phosphorylated immediately in response to DNA damage signal and subsequently trigger DNA damage repair [24]. To determine the relevant signaling pathway implicated in the PUN-evoked DNA damage in BCPAP cells, we next detected the phosphorylation levels of ATM and ATR by Western blot. As shown in Fig. 4A, PUN augmented the phosphorylation of ATM in a concentration-dependent manner. However, the phosphorylation of ATR as well as its total protein was not affected with PUN treatment, indicating that PUN-induced DNA damage repair is regulated by ATM rather than ATR. Moreover, PUN increased the level of p-ATM in a time-dependent manner (Fig. 4B). Altogether, we demonstrated that PUN induces the DDR through the activation of ATM but not ATR.

3.5. *ATM-mediated DDR is involved in PUN-induced cell death of BCPAP cells*

KU-55933, a selective ATM kinase inhibitor, was used to elucidate the role of ATM in the cell death induced by PUN. As shown in Fig. 5A, the up-regulation of p-ATM induced by PUN was markedly attenuated when combined with 10 μM KU-55933. Next, MTT assay was performed to measure the role of ATM activation in the cell viability of BCPAP cells. Punicalagin at 100 μM significantly decreased the cell viability of BCPAP cells and KU-55933 alone (5 or 10 μM) had no effect. However, 10 μM KU-55933 reversed the decreased cell viability caused by PUN significantly ($P < .05$; Fig. 5B). Taken together, ATM kinase activity is part of the process of PUN-induced cell death.

4. Discussion

Currently, bioactive compounds derived from food, such as curcumin, anthocyanin, capsaicin, and peptides, possess a lot of physiological functions, and one of the attractive roles is their anticancer effect [16,25-27]. Polyphenols are a large class of natural compounds, and among their various food sources, pomegranate has shown good anticancer activity [28]. Pomegranate contains several kinds of polyphenols, among which PUN, punicalin, gallagic acid, ellagic acid, and gallic acid form the major composition [29]. These components vary in molecular weight, but all have benzene ring, hydroxyl, and carbonyl and thus possess good solubility and hydrolysis ability. It was illustrated that PUN and ellagic acid are able to inhibit the proliferation of human oral, colon, and prostate cancer cells and induce the apoptosis of colon cancer cells [30]. As for the other 3 components, punicalin, gallagic acid, and gallic acid show strong antioxidant capacity [31-33]. The similar physicochemical properties of these compounds may be largely correlated with their analogical chemical structure.

Punicalagin, the biggest polyphenol up to date, has been elucidated as an anticancer agent in various types of tumors. For instance, PUN inhibits the cell growth of prostate cancer [2] and non–small cell lung cancer [3] through the induction of apoptosis. Moreover, we recently elucidated that PUN induces autophagic cell death, which is independent of apoptosis, in PTC BCPAP cells [13]. Given that DNA damage was one of the inducers of autophagy [11], we investigated whether PUN induces DNA damage in BCPAP cells. In this study, we demonstrated that PUN enhances the expression level of p-H2A.X in BCPAP cells. Besides, our results showed that PUN induces DNA damage independent of the DNA conformational alteration and the induction of ROS, which are involved in the process of DNA damage in some cases [20,22]. Interestingly, PUN was previously reported to protect DNA against damage exogenously [4], whereas our results showed that it caused DNA damage in BCPAP cells. These discrepancies may result from the differences in environment or experimental design, in particular the duration of DNA adduct or cell exposure to PUN. The in vivo actions of this compound need to be examined as well as any metabolite of PUN.

DNA damage usually results in the initiation of DDR. ATM- and ATR-dependent signaling pathways are the major executors in DDR, whereas our results showed that the DDR induced by PUN in PTC cells is mediated by ATM but not ATR.

DNA damage response is a double-edged sword in cancers, which consists of 2 processes: signaling transduction and DNA repair. On the one hand, it quickly recruits repair proteins to target sites to counteract the effect of drug or radiotherapy. It has been reported that ATM together with leucine zipper motif (APPL), a regulator of ATM phosphorylation, modulates DNA damage repair and consequently raises the survival of pancreatic carcinoma cells after irradiation [34]. In addition, DDR may up-regulate RAD51 to promote DNA damage repair and thereby make the prostate cancer cells be resistant to drug-induced DNA damage [35]. On the other hand, deficient DDR would probably lead to excessive DNA damage and bring about genomic instability or genetic traits changes and even result in cell death [36]. Several DNA damaging agents have been demonstrated to exert good suppression effects in cancers, among which, cisplatin, doxorubicin, and etoposide have been applied to clinical trials against thymic epithelial tumors and peripheral T-cell non–Hodgkin lymphoma [37,38]. Moreover, we have previously elucidated that curcumin caused the apoptosis through the function of DNA damage in BCPAP cells [16]. In this article, we showed that PUN-induced DDR participated in the cell death of BCPAP, which was in accordance with our hypothesis that DNA damage was involved in the process of PUN-induced cell death. Notably, positive TUNNEL spots examined after the treatment of PUN were more reminiscent of apoptosis. However, we previously showed that PUN induces BCPAP cell death independent of apoptosis but through autophagic cell death [13]. In addition, PUN induces a slight alteration in cell cycle even under the higher concentration [13]. Collectively, these may be explained by a lack of time for effective DNA damage repair, which resulted in autophagic cell death. However, the direct correlation between DDR and autophagic cell death induced by PUN has not been well clarified, which needs more efforts to probe.

In human cancers, DDR-related genes are usually depleted or mutated to escape from cell cycle arrest, which is mediated by DDR [36]. In accordance, several researches showed that the variation of *ATM* gene may contribute to the susceptibility to thyroid carcinoma in individuals [39,40]. Furthermore, the ratio of *ATM* mutation is higher in anaplastic thyroid carcinoma than in PTC [41], suggesting that *ATM* mutation is closely associated with the malignant state of thyroid cancer. Consistently, our study demonstrated that ATM kinase activity is indispensable in PUN-induced PTC cell death. However, up-regulated expressions of ATM and γH2A.X (namely p-H2A.X) were reported in thyroid cancer as well [42], hinting that ATM and γH2A.X are involved in the differentiation status of thyroid cancer. Besides, the inhibition of ATM was shown to improve the curative effect of radioactive iodine-131, indicating that ATM may promote radioresistance in thyroid cancer treatment [43]. Given the multiple roles of ATM, it requires more studies to distinguish the detailed mechanism in thyroid cancer with attention to in vivo work.

In addition, there are still some limitations in our work that need further exploration. Although we have found that PUN markedly inhibited the cell viability of BCPAP cells rather than that of normal thyroid cells Nthy-ori-3.1 (data not shown), the underlying mechanisms of the cytotoxicity selectivity of PUN have not been well investigated. Moreover, whether PUN could induce cell death of other types of thyroid cancer cells like follicular thyroid cancer or even anaplastic thyroid cancer cells is still a remained issue to be elucidated. At the same time, the upstream and downstream of ATM as well as the rationale of ROS-independent DNA damage induced by PUN (which is without DNA conformational alteration) in BCPAP cells require further investigation. Answers to these questions are necessary for fully understanding the anticancer efficacy of PUN.

Overall, our study demonstrated that PUN, as a natural compound from pomegranate, promotes cell death via triggering ATM-mediated DDR in human PTC BCPAP cells, which is independent of ROS and DNA conformational change. Our work contributes to a better understanding of the anticancer mechanisms of PUN and provides more scientific evidence for the potential medical value of natural compounds as well as imply the potential targets for PTC therapy.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81402214, 81602352, and 81422050), Research Innovation Program of academic degree postgraduates in colleges and universities in Jiangsu Province (Grant No. KYZZ16_0061), and Wuxi Health and Family Planning Commission Foundation (Grant No. Q201608). The authors report no conflicts of interest.

REFERENCES

[1] Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li HB. Resources and biological activities of natural polyphenols. Forum Nutr 2014; 6:6020–47.

[2] Adaramoye O, Erguen B, Nitzsche B, Höpfner M, Jung K, Rabien A. Punicalagin, a polyphenol from pomegranate fruit, induces growth inhibition and apoptosis in human PC-3 and LNCaP cells. Chem Biol Interact 2017;274:100–6.

[3] Li Y, Yang F, Zheng W, Hu M, Wang J, Ma S, et al. *Punica granatum* (pomegranate) leaves extract induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in non–small cell lung cancer in vitro. Biomed Pharmacother 2016;80:227–35.

[4] Aqil F, Munagala R, Vadhanam MV, Kausar H, Jeyabalan J, Schultz DJ, et al. Anti-proliferative activity and protection against oxidative DNA damage by punicalagin isolated from pomegranate husk. Food Res Int 2012;49:345–53.

[5] Hanley JP, Jackson E, Morrissey LA, Rizzo DM, Sprague BL, Sarkar IN, et al. Geospatial and temporal analysis of thyroid cancer incidence in a rural population. Thyroid 2015;25: 812–22.

[6] Davies L, Morris LG, Haymart M, Chen AY, Goldenberg D, Morris J, et al. American Association of Clinical Endocrinologists and American College of Endocrinology Disease state clinical review: the increasing incidence of thyroid cancer. Endocr Pract 2015;21:686–96.

[7] Gianoukakis AG, Flores NM, Pelletier CL, Forsythe A, Wolfe GR, Taylor MH. Treatment patterns, health state, and health care resource utilization of patients with radioactive iodine refractory differentiated thyroid cancer. Cancer Manag Res 2016;8:67–76.

[8] Mermershtain I, Glover JM. Structural mechanisms underlying signaling in the cellular response to DNA double strand breaks. Mutat Res 2013;750:15–22.

[9] Matt S, Hofmann TG. The DNA damage-induced cell death response: a roadmap to kill cancer cells. Cell Mol Life Sci 2016; 73:2829–50.

[10] Centurione L, Aiello FB. DNA repair and cytokines: TGF-beta, IL-6, and thrombopoietin as different biomarkers of radioresistance. Front Oncol 2016;6:175.

[11] Rodriguez-Rocha H, Garcia-Garcia A, Panayiotidis MI, Franco R. DNA damage and autophagy. Mutat Res 2011;711:158–66.

[12] Alexander A, Kim J, Walker CL. ATM engages the TSC2/mTORC1 signaling node to regulate autophagy. Autophagy 2010;6:672–3.

[13] Cheng X, Gao Y, Yao X, Yu H, Bao J, Guan H, et al. Punicalagin induces apoptosis-independent autophagic cell death in human papillary thyroid carcinoma BCPAP cells. RSC Adv 2016;6:68485–93.

[14] Gupta E, Kaushik S, Purwar S, Sharma R, Balapure AK, Sundaram S. Anticancer potential of steviol in MCF-7 human breast cancer cells. Pharmacogn Mag 2017;13:345–50.

[15] Zhang L, Cheng X, Gao Y, Zheng J, Xu Q, Sun Y, et al. Apigenin induces autophagic cell death in human papillary thyroid carcinoma BCPAP cells. Food Funct 2015;6:3464–72.

[16] Zhang L, Cheng X, Gao Y, Bao J, Guan H, Lu R, et al. Induction of ROS-independent DNA damage by curcumin leads to G2/M cell cycle arrest and apoptosis in human papillary thyroid carcinoma BCPAP cells. Food Funct 2016;7:315–25.

[17] Biterge B, Schneider R. Histone variants: key players of chromatin. Cell Tissue Res 2014;356:457–66.

[18] Monteiro FL, Baptista T, Amado F, Vitorino R, Jerónimo C, Helguero LA. Expression and functionality of histone H2A variants in cancer. Oncotarget 2014;5:3428–43.

[19] Talbert PB, Henikoff S. Environmental responses mediated by histone variants. Trends Cell Biol 2014;24:642–50.

[20] Ramachandran S, Temple BR, Chaney SG, Dokholyan NV. Structural basis for the sequence-dependent effects of platinum-DNA adducts. Nucleic Acids Res 2009;37:2434–48.

[21] Zhang B, Seki S, Akiyama K, Tsutsui K, Li T, Nagao K. Detection and analyses by gel electrophoresis of cisplatin-mediated DNA damage. Acta Med Okayama 1992;46:427–34.

[22] Roszkowski K. Oxidative DNA damage—the possible use of biomarkers as additional prognostic factors in oncology. Front Biosci (Landmark Ed) 2014;19:808–17.
[23] Mahl CD, Behling CS, Hackenhaar FS, de Carvalho e Silva MN, Putti J, Salomon TB, et al. Induction of ROS generation by fluconazole in *Candida glabrata*: activation of antioxidant enzymes and oxidative DNA damage. Diagn Microbiol Infect Dis 2015;82:203–8.
[24] Ray A, Blevins C, Wani G, Wani AA. ATR- and ATM-mediated DNA damage response is dependent on excision repair assembly during G1 but not in S phase of cell cycle. PLoS One 2016;11:e0159344.
[25] Anandakumar P, Kamaraj S, Jagan S, Ramakrishnan G, Asokkumar S, Naveenkumar C, et al. The anticancer role of capsaicin in experimentallyinduced lung carcinogenesis. J Pharmacopuncture 2015;18:19–25.
[26] Chakrabarti S, Jahandideh F, Wu J. Food-derived bioactive peptides on inflammation and oxidative stress. Biomed Res Int 2014;2014:608979.
[27] Lin BW, Gong CC, Song HF, Cui YY. Effects of anthocyanins on the prevention and treatment of cancer. Br J Pharmacol 2017; 174:1226–43.
[28] Panth N, Manandhar B, Paudel KR. Anticancer activity of punica granatum (pomegranate): a review. Phytother Res 2017;31:568–78.
[29] Madrigal-Carballo S, Rodriguez G, Krueger CG, Dreher M, Reed JD. Pomegranate (*Punica granatum*) supplements: authenticity, antioxidant and polyphenol composition. J Funct Foods 2009;1:324–9.
[30] Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005;16:360–7.
[31] Wang Y, Zhang H, Liang H, Yuan Q. Purification, antioxidant activity and protein-precipitating capacity of punicalin from pomegranate husk. Food Chem 2013;138:437–43.
[32] Johanningsmeier SD, Harris GK. Pomegranate as a functional food and nutraceutical source. Annu Rev Food Sci Technol 2011;2:181–201.
[33] Asnaashari M, Farhoosh R, Sharif A. Antioxidant activity of gallic acid and methyl gallate in triacylglycerols of Kilka fish oil and its oil-in-water emulsion. Food Chem 2014;159: 439–44.
[34] Hennig J, McShane MP, Cordes N, Eke I. APPL proteins modulate DNA repair and radiation survival of pancreatic carcinoma cells by regulating ATM. Cell Death Dis 2014;5: e1199.
[35] Wu M, Wang X, McGregor N, Pienta KJ, Zhang J. Dynamic regulation of Rad51 by E2F1 and p53 in prostate cancer cells upon drug-induced DNA damage under hypoxia. Mol Pharmacol 2014;85:866–76.
[36] Tian H, Gao Z, Li H, Zhang B, Wang G, Zhang Q, et al. DNA damage response—a double-edged sword in cancer prevention and cancer therapy. Cancer Lett 2015;358:8–16.
[37] Thomas A, Rajan A, Szabo E, Tomita Y, Carter CA, Scepura B, et al. A phase I/II trial of belinostat in combination with cisplatin, doxorubicin, and cyclophosphamide in thymic epithelial tumors: a clinical and translational study. Clin Cancer Res 2014;20:5392–402.
[38] Mahadevan D, Unger JM, Spier CM, Persky DO, Young F, LeBlanc M, et al. Phase 2 trial of combined cisplatin, etoposide, gemcitabine, and methylprednisolone (PEGS) in peripheral T-cell non–Hodgkin lymphoma: Southwest Oncology Group Study S0350. Cancer 2013;119:371–9.
[39] Xu L, Morari EC, Wei Q, Sturgis EM, Ward LS. Functional variations in the ATM gene and susceptibility to differentiated thyroid carcinoma. J Clin Endocrinol Metab 2012;97:1913–21.
[40] Wójcicka A, Czetwertyńska M, Świerniak M, Długosińska J, Maciag M, Czajka A, et al. Variants in the ATM-CHEK2-BRCA1 axis determine genetic predisposition and clinical presentation of papillary thyroid carcinoma. Genes Chromosomes Cancer 2014;53:516–23.
[41] Choi M, Kipps T, Kurzrock R. ATM mutations in cancer: therapeutic implications. Mol Cancer Ther 2016;15:1781–91.
[42] Hu JL, Hu SS, Hou XX, Zhu X, Cao J, Jiang LH, et al. Abnormal expression of DNA double-strand breaks related genes, ATM and gammaH2AX, in thyroid carcinoma. Int J Endocrinol 2015;2015:136810.
[43] Lyckesvärd MN, Kapoor N, Ingeson-Carlsson C, Carlsson T, Karlsson JO, Postgård P, et al. Linking loss of sodium-iodide symporter expression to DNA damage. Exp Cell Res 2016;344: 120–31.