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Punicalagin induces apoptosis-independent autophagic cell death in human papillary thyroid carcinoma BCPAP cells

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Thyroid cancer is the most common endocrine carcinoma with increased incidence worldwide. Punicalagin is a tannin in pomegranate and contributes to a variety of physiological and pathological processes including inflammation, immunity and cancer. However, the exact role of punicalagin played in thyroid cancer treatment has not been elucidated yet. Our study shows that punicalagin decreased the viability of thyroid cancer cell line BCPAP. Punicalagin treatment did not influence the nuclear fragmentation or chromatin condensation and cell cycle distribution of BCPAP cells. Besides, no caspase-3 and PARP cleavage was observed after punicalagin treatment. These characteristics integrally indicated that punicalagin initiated a non-apoptotic cell death in BCPAP cells. Autophagy is a response of cancer cells to various anticancer therapies. In the present study, punicalagin treatment resulted in marked autophagy induction as evidenced by an increase in LC3-II conversion and beclin-1 expression, and increased in p62 degradation. In addition, punicalagin-induced cell death was significantly inhibited by autophagy inhibitors 3-methyladenine. Moreover, the punicalagin activates the MAPK and inhibits the mTOR signaling pathways to promote the process of autophagy. Taken together, our results provide evidences for the antitumor effect of punicalagin which is considerably linked to its ability to induce autophagic cell death.

1. Introduction

Thyroid cancer is the most common endocrine carcinoma and its incidence steadily increases three times in the past decade.[1] The most common type of thyroid cancer is papillary thyroid cancer (PTC) which accounts for almost 80% of all thyroid cancers. Conventional treatment strategies of thyroid cancer including surgical thyroidectomy, hormone suppression and radiotherapy which generally has a good prognosis.[2] However, there are still a subset of patients with PTC, especially those patients harboring *BRAF* mutations and *TERT* promoter mutation, who have poorer clinic-pathological outcomes and higher risk ratio of recurrence.[3,4] Unveiling the molecular process of thyroid cancer cell death will provide unprecedented opportunities for thyroid carcinoma treatment and novel drug development.

Autophagy is a self-digestion process to maintain the homeostasis between biosynthesis and turnover[5] and it is evolutionary conserved in all eukaryotic cells.[6] Moderate autophagy leads to cell survival, however, excessive autophagy induces cell death. Autophagy, termed as a type II programmed cell death, was first discovered as a protective mechanism from starvation.[7] Apart from apoptosis, autophagy can act independently to induce the cell death which referred to autophagic cell death.[8] Recent studies uncover the multiplicity roles of autophagy played in a wide range of pathology and physiology pathways including inflammation,[9] immunity and cancer.[10,11] Besides, it has been reported that autophagy plays a vital role involved in the development and progression of thyroid cancer.[12,13] The loss of autophagy is tightly conjugated with malignant thyroid cancer which presented in a more aggressive form.[12] Besides, active autophagy predisposes to high uptake and to effective clinical responses to radioactive iodine (RAI) therapy.[14] All these studies confirm the indispensable role of autophagy in thyroid cancer therapy.

Natural products are invaluable source for the discovery of various therapeutic agents. Punicalagin (2,3-hexahydroxydiphenoylgallagyl-D-glucose, PUN) is a kind of tannins which is abundant in the peels and seeds of pomegranate.[15] The

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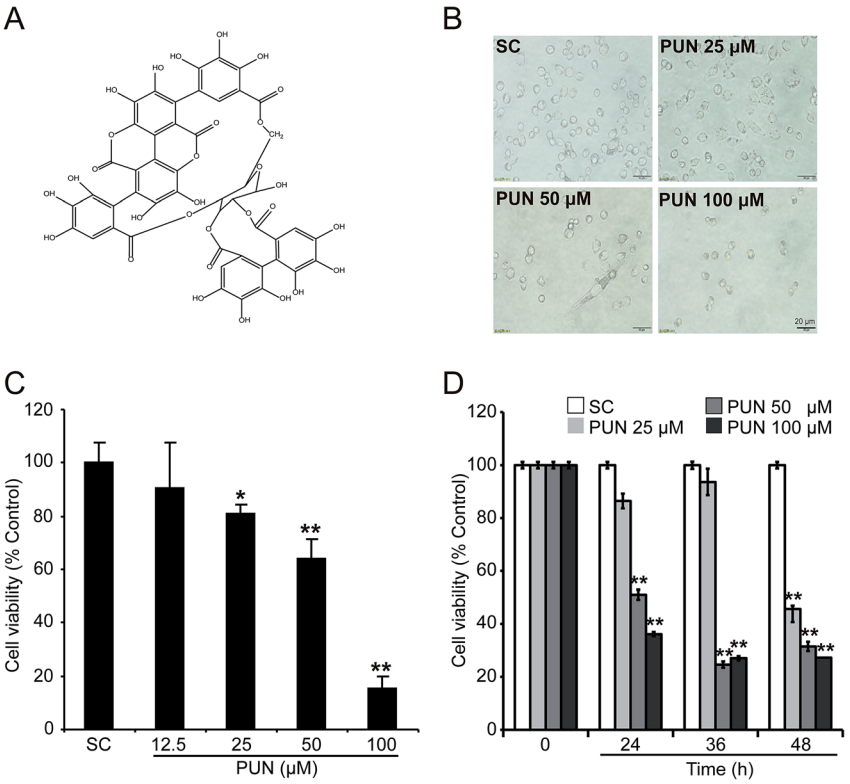

Fig. 1 Punicalagin inhibits the cell viability of BCPAP cells. (A) Chemical structure of punicalagin. (B) BCPAP cells were treated with different doses (12.5, 25, 50 and 100 μM) of punicalagin for 24 h, and the morphological alteration was examined by microscope (Olympus IX51, Japan). Scale bar: 20 μm. (C) After cells were treated with punicalagin at different concentrations (12.5, 25, 50 and 100 μM) for 24 h, the cell viability was analyzed by MTT assay. *$P < 0.05$, **$P < 0.01$. (D) BCPAP cells were treated with different concentrations of punicalagin (25, 50, 100 μM) for 24, 36 and 48 h, and the cell viability was expressed as mean ± SEM. **$P < 0.01$. SC, solvent control.

molecular structure of punicalagin is a large hydrolysable polyphenolic molecules with complex ring structures (Fig. 1A). Punicalagin possesses a broad range of pharmacological activities, including anti-atherosclerosis,[16] anti-inflammation,[17] anti-viral,[18] anti-fungal[19] and anti-cancer activity.[20] It has been reported that punicalagin induced both apoptosis and autophagy in U87 glioma cells.[21] Our previous study showed that another natural compound, curcumin, induced ER-stress and led to the cell death of thyroid cancer cells.[22] However, the detailed mechanisms underlying the anti-cancer effects of these remedies, including punicalagin, on thyroid cancer cells and the potential clinical therapy of thyroid cancer has not been elucidated yet.

Herein, we demonstrate that punicalagin induces the process of autophagic cell death in BCPAP cell lines which harbored *BRAF* and *TERT* mutations.

2. Material and methods

2.1 Chemicals and reagents

Punicalagin (HPLC ≥ 98%) was purchased from klamar-reagent (Shanghai puzhen Biotechnology Co., Ltd). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sangon (Shanghai, China). Propidium iodide (PI) and bafilomycin A_1 (Baf A_1) were purchased from Enzo Life Sciences. Acridine orange hydrochloride hydrate and 3-methyladenine (3-MA) were purchased from Sigma Aldrich. The used antibodies were diluted at 1 : 1000 and listed as follows: anti-LC3 antibody, anti-p62 antibody were purchased from Sigma Aldrich. Anti-PARP antibody, anti-β-actin antibody and anti-beclin-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase3 antibody, anti-JNK, anti-phosphate-JNK (Thr183/Tyr185), anti-ERK, anti-phosphate-ERK (Thr202/Tyr204), anti-p38, anti-phosphate-p38 (Thr180/Tyr182), anti-p70S6, anti-phosphate-p70S6 (T389), anti-S6, anti-phosphate-S6 (Ser235/236) and anti-phospho-4E-BP1 (Thr37/46) antibodies were purchased from Cell Signaling Technology. All of the other chemicals were of the highest analytical grade and purchased from common sources.

2.2 Cell culture and treatments

The BCPAP cell line, derived from a human papillary thyroid carcinoma, was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cells were cultured in RPMI 1640 (Gibco, USA) containing 10% calf serum (Sijiqing, Hangzhou, China), 100 U ml^{-1} penicillin, and 100 mg l^{-1} streptomycin. The cells were incubated in a humidified incubator (Thermo Electron Corporation, USA) with atmosphere of 5% CO_2 at 37 °C.

Punicalagin was dissolved in methanol at 50 mM as a stock solution and stored at 4 °C until diluted before use. Unless otherwise indicated, cells were treated with various concentrations (12.5 to 100 μM) of punicalagin for 24 h. The solvent control contains an equivalent amount of methanol corresponding to the highest used concentration of punicalagin (final concentration, 0.2% methanol). Bafilomycin A_1 was stored in DMSO at 2 mM and diluted to a final concentration of 25 nM. 3-MA was diluted in double distilled water to a final concentration of 10 mM when used. H_2O_2 was freshly prepared diluted from 30% stock solution to a working concentration at 200 μM prior to each experiment.

2.3 Cytotoxicity assays

BCPAP cells were seeded in 96-well plates (Costar, Corning Incorporated, USA) at a density of 1.0×10^4 cells per well overnight. The cells were then treated with punicalagin at different concentrations varied from 25 μM to 100 μM for 24 h. Then 20 μl of MTT storage solution was added to each well at a final concentration of 1 mg ml^{-1}. After additional incubation for 4 h, the supernatant was discarded and the crystal violet deposits were dissolved in 150 μl of DMSO. After vortex for 15 min to completely dissolve the precipitate, the absorbance at 490 nm was measured with a microplate reader (μ Quant, BIO-Tek, USA).

2.4 Protein extraction and western blotting analysis

The cells were collected after treatment with punicalagin for 24 h. After cells were washed with PBS, they were incubated in ice-cold lysis (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) Nonidet P-40, 0.02% (w/v) sodium azide) within protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF). Cells were kept on ice for 15 min and shake per 2 min and centrifuged to yield whole cell lysates. For western blotting, proteins were separated with 15% polyacrylamide gels and then electrotransferred onto a nitrocellulose filter membrane. After that, the membrane was blocked in 5% nonfat milk solution for at least 30 min. The membrane was incubated with the indicated primary antibody overnight at 4 °C, and then washed and finally incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for additional 1 h. After washing by TBST for three times, the protein bands were then detected with an enhanced chemiluminescence (ECL) substrate solution (Beyotime Biotechnology, China).

2.5 Hoechst 33342 and propidium iodide staining

Hoechst 33342 and propidium iodide (PI) staining was used to observe the apoptotic/necrotic morphology of BCPAP cells. In brief, 1×10^5 cells were seeded into 6-well plates and incubated for 24 h with punicalagin. After incubation, 1 μl of PI (50 μg ml^{-1}) and 5 μl of Hoechst 33342 (10 mg ml^{-1}) were added to each well and incubated for 30 min at 37 °C. Cells were then investigated using Leica fluorescence microscope and photos were captured at five random fields.

2.6 Measurement of cell cycle by flow cytometry

Cells were treated with different concentrations of punicalagin for 24 h and stained with 50 μg ml^{-1} of propidium iodide (PI) to determine the DNA contents. Data from 20 000 cells per sample were recorded and percentages of cells within G0/G1, S, and G2/M cell cycle phases were measured by FlowJo software.

2.7 Acridine orange staining

After treatment with different doses of punicalagin, the BCPAP cells were stained with acridine orange for 30 min. Then cells were washed twice and detected by flow cytometry. The numbers of autophagic vacuoles (AVOs) were measured in FL1-H and FL3-H channels and the intensity plots in each region were quantified by BD CellQuest software.

2.8 Statistical analysis

All the data are represented as means ± S.D. of triplicate experiments. One-way ANOVA was used to determine the statistical significance and comparison between groups were made using Student's *t*-test. Results were considered statistically significant when P value ≤ 0.05.

3. Results

3.1 Punicalagin inhibits the cell viability of BCPAP cells

First, the cell morphology of BCPAP cells was observed after treatment with punicalagin (25, 50, 100 μM) for 24 h. As shown in Fig. 1B, the amount of cell number decreases gradually with the increased concentration of punicalagin and a subset of the punicalagin-treated cells enlarged and contained vacuoles. Next, the viability of BCPAP cells after punicalagin treatment was measured by MTT assay. As shown in Fig. 1C, after treatment with punicalagin for 24 h, the cell viability of BCPAP cells significantly decreased. Compared with control group, the cell viability was significantly reduced to 90.7 ± 16.7%, 80.9 ± 3.5%, 64.1 ± 7.4% and 15.3 ± 4.2% at 12.5, 25, 50 and 100 μM, respectively. Furthermore, BCPAP cells were exposed to punicalagin at different times and dosages. As shown in Fig. 1D, significant inhibitory effect on proliferation were observed under 50 and 100 μM of punicalagin exposure for 24, 36 or 48 h incubation, while a significant reduction can be detected under 25 μM for 48 h incubation. These results showed that punicalagin induced cell death of BCPAP cells both in a time- and concentration-dependent way.

3.2 Punicalagin fails to induce the apoptosis of BCPAP cells

Apoptosis is a classically recognized form of cell death and its mechanism is well studied.[23] A series of changes occurred in apoptotic cells at the morphological and molecular levels which were classically termed as chromatin condensation, DNA fragmentation, mitochondrial alteration and the activation of caspase family members.[24] To further investigate the probable involvement of punicalagin in triggering the apoptotic cell death of BCPAP cells, Hoechst/PI double staining assay was used to evaluate the apoptotic cells. In this assay, condensed

chromatin of apoptotic cells would be stained with Hoechst dye and spotted as intensively blue dots. Whereas late phase apoptotic cells or necrosis were stained as red, since PI could only penetrate damaged cell membranes. As shown in Fig. 2A, no bright blue, condensed or fragmented nuclei stain displayed in punicalagin-treated cells, indicating that punicalagin do not induce the cell apoptosis of BCPAP. To be pointed out, only few red staining cells were spotted with higher concentration of punicalagin treatment at 100 μM, which ruled out the possibility of punicalagin-mediated necrosis. To further confirm these results, PARP cleavage and caspase activation, which serves as common mediators of apoptotic cell death, were also detected. Consistent with no appreciable apoptotic bodies detected in Fig. 2A, neither cleaved form of PARP nor caspase-3 activation can be detected after punicalagin treatment. However, H_2O_2, which used as a positive control for apoptosis,[25] induced an obvious caspase-3 cleavage in BCPAP cells (Fig. 2B). The distribution of cell cycle is closely related with cell proliferation characterized as G0/G1, S and G2/M phases.[26] Damaged DNA will be degraded into many small fragments.[27] Thus, cells undergoing apoptosis will cause the emergence of sub-G1 group, a group contained of DNA smaller than diploid. Next, we examined the cell cycle distribution of BCPAP after punicalagin treatment by PI staining. As illustrated in Fig. 2C, no sub-G1 peaks could be observed under different dosages of punicalagin exposure, even under the highest concentration at 100 μM. Moreover, punicalagin treatment did not alter the cell cycle distribution of BCPAP cells compared to the control group. All these results suggest that punicalagin does not induce the apoptosis of BCPAP cells.

3.3 Punicalagin induces autophagy in BCPAP cells

It has been reported that autophagy is required for the cancer cell death once apoptosis is disabled.[28] Different autophagy-related genes (*ATG*) participate in the process of autophagy which form a complex and fine regulatory networks.[29] Microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is the most widely used marker for

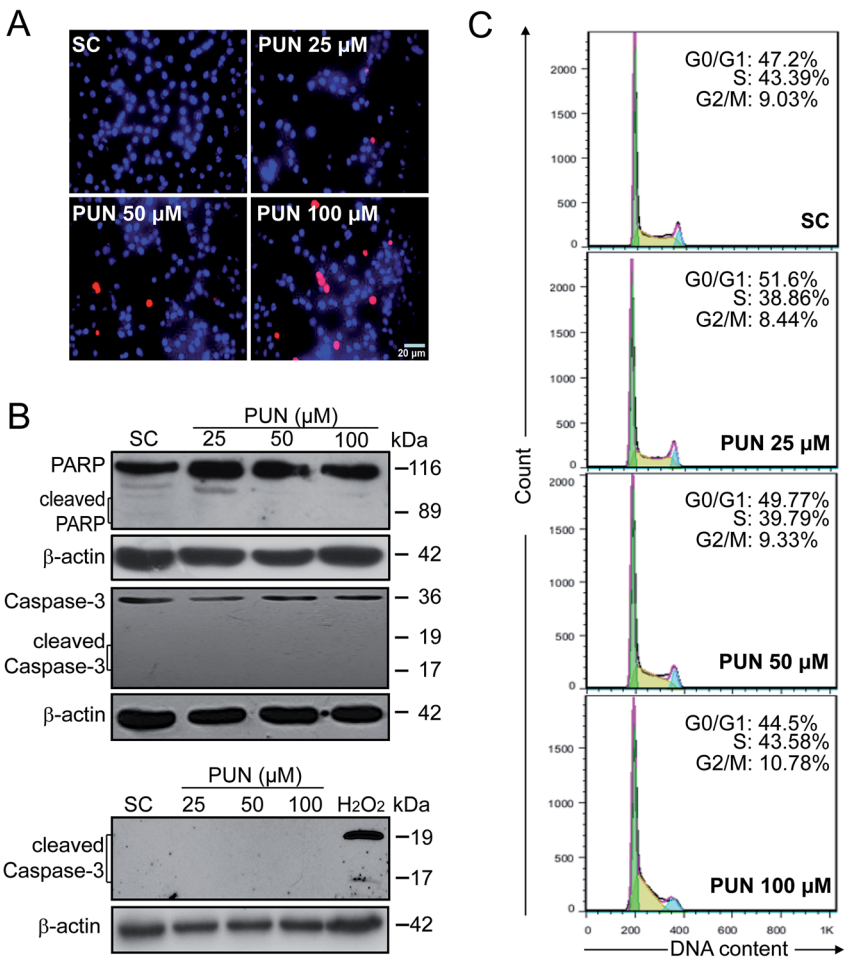

Fig. 2 Punicalagin does not induce apoptosis of BCPAP cells. (A) After treatment with different dosages of punicalagin for 24 h, BCPAP cells were stained with PI (10 μg ml^{-1}) and Hochest 33342 (10 μg ml^{-1}) and then observed under a fluorescence microscope. Scale bar, 20 μm. (B) BCPAP cells were treated with punicalagin for 24 h and then the whole cell proteins were extracted. The apoptotic molecular biomarkers, caspase-3 and PARP cleavage, were measured by western blot analysis. H_2O_2 (200 μM) was used as a positive control for apoptosis. (C) After incubation with punicalagin for 24 h, the cells were stained with propidium iodide (PI, 10 μg ml^{-1}) followed by analyses of cell cycle distribution using flow cytometry. The data was analyzed by Flowjo software with Watson model.

autophagosomes.[7] Once autophagy is induced, LC3 is conjugated to phosphatidylethanolamine (PE) and then cleaved by upstream ATG4B. Therefore, the nonlipidated and lipidated form of LC3 could be detected by immune-blotting and termed as LC3-I and LC3-II respectively. Generally, the induction of autophagy resulted in the accumulation of LC3-II. P62 is a direct target of LC3 and thus the clearance of p62 also serves as a marker for autophagy.[30] After BCPAP cells were treated with different concentrations of punicalagin for 24 h, a substantial, dose-dependent increase in LC3-II expression as well as a reduction in p62 levels were detected (Fig. 3A). The protein level of beclin-1 which is involved in the initiation of autophagy and phagosome formation was also measured. As shown in Fig. 3A, apparent up-regulation of beclin-1 protein levels were detected after punicalagin treatment compared to the solvent control. To further confirm the induction of autophagosomes by punicalagin, we probed the BCPAP cells with acridine orange staining to detect the formation of autophagic vacuoles (AVOs). As shown in Fig. 3E, punicalagin treatment dose-dependently increased the number of AVOs in BCPAP cells. Compared with

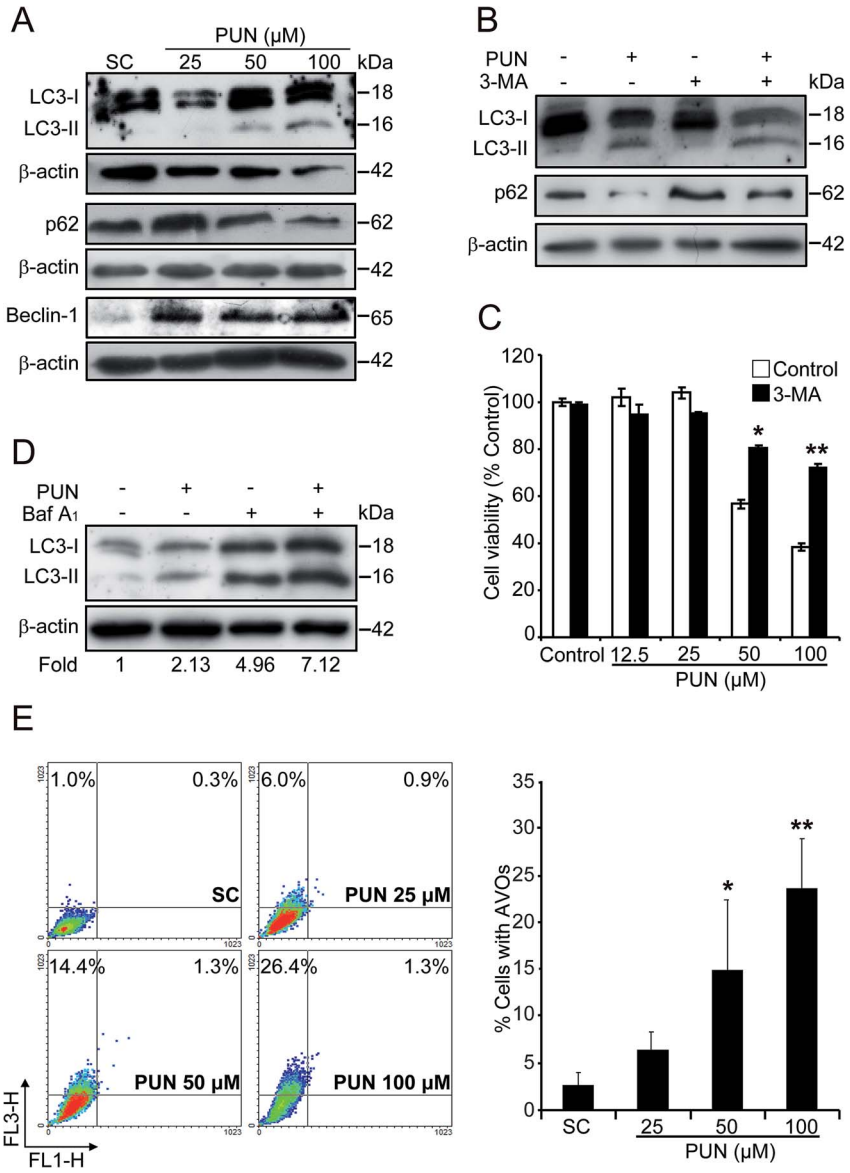

Fig. 3 Punicalagin induces autophagic cell death in BCPAP cells. (A) Cells were treated with punicalagin (25, 50 and 100 μM) for 24 h and the LC3, p62 and beclin-1 protein levels were analyzed by western blotting. β-Actin was used as the loading control. (B and C) BCPAP cells were incubated with punicalagin in the presence or absence of 3 MA (10 mM) for 24 h. The levels of LC3 and p62 were determined by western blot in (B) and the cell viability was measured by MTT assay in (C). *$P < 0.05$, **$P < 0.01$ *vs.* control group. (D) Punicalagin increases autophagy flux of BCPAP cells. Cells were incubated with punicalagin in the presence or absence of bafilomycin A_1 (100 nM) for 24 h and the LC3 conversion was analyzed by western blotting. (E) Punicalagin enhances AVOs (autophagic vacuoles) formation of BCPAP cells. After treatment with different doses of punicalagin for 24 h, cells were stained with acridine orange (1 μg ml^{-1}) and examined by flow cytometry. AVOs were quantified using BD CellQuest software and the representative result of three independent assays was shown. Quantification of AVOs formation in BCPAP cells treated with punicalagin was shown. All results were presented as means ± SEM. *$p < 0.05$; **$p < 0.01$.

control group, the percentage of acridine orange-stained cells in 50 and 100 μM of punicalagin treatment group were dramatically increased from 2.6 ± 1.5% to 14.8 ± 7.5% and 23.6 ± 5.2%, respectively.

Autophagy is a dynamic process including the formation of autophagosomes and fusion with lysosomes.[7] As expected, 3-MA, an autophagy inhibitor which inhibits the initiation of autophagy,[30] suppressed punicalagin-induced autophagy as evidenced by the decrease in the expression of LC3-II and blockage in p62 degradation (Fig. 3B). Furthermore, 3-MA restored the punicalagin-induced cell death which indicated that the autophagy induced by punicalagin acted as a death signal for BCPAP cells (Fig. 3C). On the other hand, bafilomycin A_1 (Baf A_1), which targets later phase of autophagy by inhibiting acidification inside the lysosome, was commonly used to evaluate the autophagy flux.[30] As shown in Fig. 3D, in the presence of bafilomycin A_1, punicalagin can still enhance the LC3-II protein level. Taken together, these data indicate that punicalagin induces autophagic cell death of BCPAP cells.

3.4 Punicalagin induces autophagy through the activation of MAPK pathway and the inhibition of mTOR pathway

It has been proved that MAPK signaling pathways plays a vital role in autophagy regulation.[31] Three major MAP kinases have been identified, *i.e.*, ERK 1/2, JNK, and p38 kinases.[31,32] As shown in Fig. 4A, incubation of BCPAP cells with punicalagin dose-dependently induced ERK1/2 and p38 phosphorylation, but not JNK phosphorylation. These data indicated that ERK and p38 activations are required for induction of autophagy in punicalagin-treated BCPAP cells whereas JNK are not involved. On the other hand, mTOR signaling pathway serves as a negative mediator of autophagy, and the inhibition of mTOR caused autophagy activation. p70S6, S6 and 4E-BP1 are three main downstream regulators of mTOR signaling pathway. As expected, the punicalagin dose-dependently inhibited the protein levels of phosphorylated p70S6, S6 and 4E-BP1 (Fig. 4B). Overall, punicalagin induces autophagy by regulation of MAPK and mTOR signaling pathway.

4. Discussion

It has been proved that extracts derived from pomegranate, including flavonoids, ellagitannins and the unique ellagitannin, punicalagin, have potential chemotherapy effects.[15] Punicalagin is a polyphenol that is concentrated in both fruit and seeds[15] and has been demonstrated as the main ingredient accounted for the anti-oxidant role of pomegranate juice.[17] Moreover, punicalagin has been found to exert its important role in the prevention of cancer, for instance, colon[33] and glioma cancer progression.[21] Punicalagin is a big polyphenol bomb and it can be easily hydrolyzed to various small molecules.[15] It has been reported that ellagic acid, one of the hydrolysis products of punicalagin, also exerts anti-mutagenic and anti-proliferative potential in cancer cells.[34] In our study, we found that punicalagin induced autophagy in thyroid cancer. It is interesting to note that, ellagic acid has been reported to inhibit starvation-induced autophagy.[35] The distinct roles of punicalagin and its hydrolysis products in regulating autophagy may be attributed to their different chemical structure. Further studies are necessary to distinguish the structure–function properties of punicalagin.

In the present study, we first confirmed that punicalagin inhibited the growth of thyroid cancer cell BCPAP in a time-

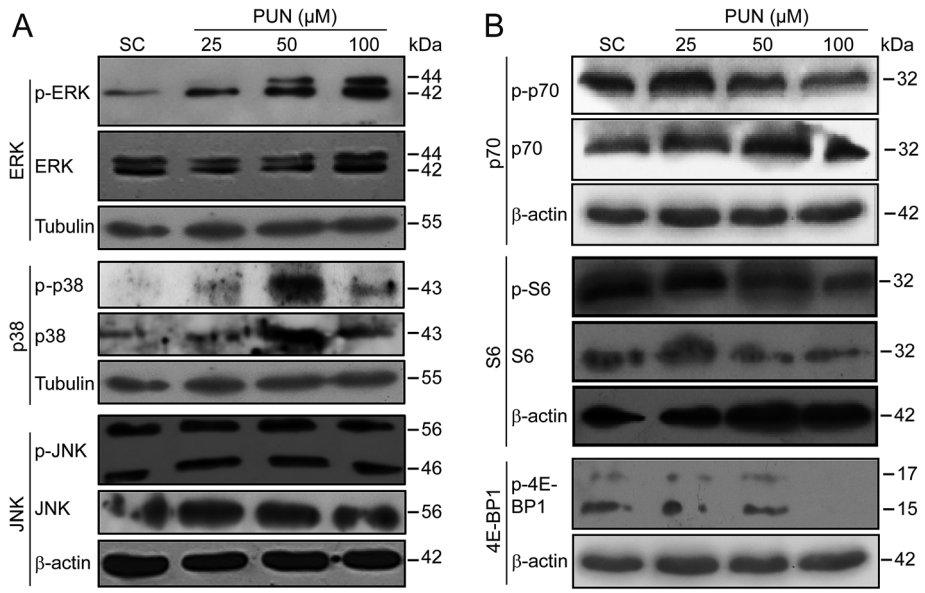

Fig. 4 Punicalagin induces autophagy through the activation of MAPK pathway and the inhibition of mTOR pathway. (A) After cells were treated with punicalagin at different concentration (25, 50, 100 μM) for 24 h, the whole cell lysis was extracted and western blotting was used for detecting the phosphorylated and total p38, JNK, ERK1/2 levels. (B) The phosphorylated and total protein level of p70, S6 and 4E-BP1 were detected. β-Actin were used as a loading control.

and dosage-dependent manner (Fig. 1). Apoptosis is the most well studied pathway to induce the cell death. Hence, we examined whether punicalagin could trigger the apoptosis of BCPAP cells (Fig. 2). However, both PI/Hoechst staining and molecular marker of cell apoptosis examinations confirmed that punicalagin-treated cells showed no typical apoptosis features. Although it has been reported that pomegranate fruit extract induces apoptosis in many other tumor types, *i.e.*, breast cancer,[36] prostate cancer[37] and colon cancer,[38] our results excluded the possibility of punicalagin inducing cell death of thyroid cancer cells *via* apoptosis. Elsewhere, it has been reported that punicalagin targets cyclin E to induce the cell cycle arrest at the G2/S checkpoint in many cancer cells.[21] However, our results showed that punicalagin did not alter the cell cycle distributions of thyroid cancer cells. These discrepancy might represent cellular context-specific functions of punicalagin.

Autophagy is considered as a second way to cause cell death even its role was first illustrated as a protection method to against nutrition deprivation.[5] Indeed, previous study reported that punicalagin induced autophagy and protected cultured syncytiotrophoblasts from apoptosis.[39] However, unlike the apoptosis-inhibition and pro-survival function of punicalagin-induced autophagy which was proved by Y. Wang *et al.*,[39] our results showed that punicalagin was able to induce an apoptosis-independent autophagic cell death in a different cell line, BCPAP cells. It is well-characterized that autophagy is highly cell type- and context-dependent. Especially in the pathogenesis of cancer, autophagy is thought to be a double-edged sword, as it functions to promote or inhibit cancer development and progression in a state- and context-dependent manner. The function of autophagy in cancer is complex since the moderate autophagy will supply amino acids, fatty acids and nucleotides for tumor progression while excessive autophagy may lead to the cancer cell death.[11] It is an intriguing method to induce autophagic cell death in cancer therapy since many carcinomas are resistant to pro-apoptotic therapies.[40] Therefore, our study on the autophagy-inducing effect of punicalagin may provide an alternative effective strategy to improve thyroid cancer therapy.

It has been reported mutation in autophagy-related genes, such as *ATG5* single nucleotide polymorphisms rs2245214 are more susceptible to thyroid carcinoma.[41] Furthermore, accumulating studies showed that the dysregulation of the major autophagy components are closely related to thyroid tumor progression. The downregulation of beclin-1 has been found in PTC patients with lymph node metastasis.[42] Moreover, a recent study showed that LC3-II levels of thyroid tissue and PTC samples remained at baseline.[43] Although the activity of autophagy may not consistent with the clinical pathologies, it has been clearly indicated that high autophagy activity is positively correlated with the response rate of radioiodine therapy after thyroidectomy.[44] Besides the surgical thyroidectomy, radiotherapy has been viewed as a most effective method to treat thyroid cancer. The sodium iodide symporter (NIS) mediates the uptake of radioiodine by the thyroid follicular cell. It has been reported that the downregulation of NIS is correlated with the low autophagy activity in thyroid tumors.[14] Thus, the induction of autophagy may be a promising target for thyroid cancer therapy. It has been reported that synergistic activation of autophagy with RAD001 enhances the chemosensitivity and radiosensitivity of PTC.[45] Our results demonstrated that punicalagin induced autophagy in BCPAP cells as evidenced by LC3-II accumulation and autophagic vacuoles formation (Fig. 3), which hinted that punicalagin may be useful in improving thyroid cancer therapy by targeting autophagy.

Dedifferentiated thyroid cancer are more likely resistant to radiotherapy, which markedly decreases the prognosis of thyroid cancer patients. Restoring thyroid cancer cells unique ability to concentrate iodine has become an exciting avenue of research.[46] Multiple drugs have been probed for their potential to induce re-differentiation of thyroid cancer cells.[47–49] Among which mTOR inhibitor[50] and PI3K-inhibitor have been reported to restore the ability of thyroid cell to uptake ^{131}I.[12] It is well-known that the Akt/mTOR pathway negatively regulates autophagy, whereas the ERK1/2 pathway positively regulates autophagy.[51] The efficacy of mTOR inhibitor in enhancing ^{131}I uptake further confirmed the notion that targeting the key regulators of autophagy might raise an effective way to solve the radio-iodine resistant in thyroid cancer therapy. In our study, we confirmed that punicalagin induced autophagy of thyroid cancer cells by activation of the MAPK pathway as well as inhibition of the mTOR pathway (Fig. 4). However, the effects of punicalagin on the dedifferentiation of thyroid cancer cells as to the thyroid-specific gene expression by targeting autophagy remains to be clarified.

5. Conclusions

Our data demonstrated that punicalagin inhibited the growth of human thyroid cancer cells by inducing autophagic cell death but not apoptosis *via* the activation of the MAPK pathway and the inhibition of the mTOR pathway. Our results provide a new perspective and promising pharmacology for thyroid carcinoma therapy.

Conflict of interest

The authors declare no conflicts of interest.

Abbreviations

ATG	Autophagy-related genes
AVOs	Autophagic vacuoles
Baf A1	Bafilomycin A1
LC3	Microtubule-associated protein light chain 3 (LC3)
NIS	Sodium iodide symporter
PE	Phosphatidylethanolamine
PTC	Papillary thyroid cancer
PUN	2,3-Hexahydroxydiphenoylgallagyl-D-glucose, punicalagin
RAI	Radioactive iodine

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