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The natural product peiminine represses colorectal carcinoma tumor growth by inducing autophagic cell death

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ABSTRACT

Autophagy is evolutionarily conservative in eukaryotic cells that engulf cellular long-lived proteins and organelles, and it degrades the contents through fusion with lysosomes, via which the cell acquires recycled building blocks for the synthesis of new molecules. In this study, we revealed that peiminine induces cell death and enhances autophagic flux in colorectal carcinoma HCT-116 cells. We determined that peiminine enhances the autophagic flux by repressing the phosphorylation of mTOR through inhibiting upstream signals. Knocking down ATG5 greatly reduced the peiminine-induced cell death in wild-type HCT-116 cells, while treating Bax/Bak-deficient cells with peiminine resulted in significant cell death. In summary, our discoveries demonstrated that peiminine represses colorectal carcinoma cell proliferation and cell growth by inducing autophagic cell death.

1. Introduction

Autophagy is an evolutionarily conserved pathway that delivers cellular contents to lysosomes for degradation [1]. It is an important regulatory process in eukaryotic cells that plays a critical function in the maintenance of cellular homeostasis and antagonization of metabolic stress [2,3]. Double-membrane vesicles, autophagosomes, are formed during autophagy, and these engulf cytoplasm and organelles. This is followed by fusion with lysosomes, and then the proteins and organelles are finally degraded and the obtained products are recycled as new building blocks for biomolecule synthesis [4]. Autophagy is extensively involved in numerous physiological and pathological processes, including adipogenesis, starvation and tumorigenesis, etc. [5–9]. Being a "double-edged sword", autophagy provides "recycled" energy for rapid cell growth under biological stress, on one side [10]. While on the other, the uncontrolled degradation of cytoplasmic contents by excessive autophagy is likely to be lethal for cells [11]. For instance, activation of autophagy contributes to the tolerance of nutrient deprivation in colorectal carcinoma [12]. However, dimethyl cardamonin (DMC)-induced autophagy was demonstrated to significantly suppress cell proliferation through a G2/M phase cell-cycle delay and it causes cell death in colorectal carcinoma cell lines [13].

Autophagic cell death, also called type II cell death, is an alternative programmed cell death (PCD) pathway other than apoptosis (type I cell death), which has also been validated in colorectal carcinoma [13–16]. Autophagy exists commonly during cell death under stress, but not all cell death where autophagy is present can be referred to as autophagic cell death, as cell death in many circumstances is accompanied by protective autophagy. Cells deficient in Bax and Bak, proapoptotic members of the Bcl-2 family that are essential for releasing apoptosis proteins, do not undergo apoptosis when exposed to apoptotic stimuli [17–19]. When alternative pathways were activated, these cells experienced cell death. Studies revealed that numerous double-membrane vesicles were formed in Bax/Bak-deficient cells that undergo cell death [20]. Moreover, the distribution of GFP-LC3B puncta was observed, which confirmed the formation of autophagosomes that implicated autophagic cell death [20].

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Peiminine is a natural compound extracted from *Fritillaria thunbergii*, which is widely used in Traditional Chinese Medicine for the treatment of various diseases, including cancer [21]. In this study, we showed that peiminine induced phenomenal autophagy activation and significant tumor growth repression in colorectal carcinoma cells. Our data revealed that a peiminine treatment significantly induces autophagy by activating the autophagy flux through the AMPK and PI3K/Akt pathways. By determining the cell viability level in ATG5 knock-down cells and Bax/Bak-deficient HCT-116 cells, we demonstrated that peiminine induced cell death was independent of apoptosis, at least partially. Together, our result revealed the autophagy-inducing capability of peiminine and indicated that the tumor repressive effect was mediated by autophagic cell death.

2. Materials and methods

2.1. Cell culture and transfection

The colorectal carcinoma cell line HCT-116 was cultured with Dulbecco's modified Eagle's medium supplied with 10% fetal bovine serum in a 5% CO_2 humidity incubator at 37 °C. Cells were subcultured when they reached 90% confluence. A chemically synthesized siRNA duplex was transfected into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

2.2. RNA isolation, reverse transcription and quantitative real-time PCR

Post transfection (24 h) cells were collected and after a wash in 1 × PBS they were supplied with RNAiso (Takara). The total RNA was then isolated and quantified following the manufacturer's protocol. First strand cDNA was reverse transcripted using a random primer by M-MLV (Takara). Quantitative real-time PCR was performed using SYBR Master Mix (TOYOBO) with specific primers on an AB7300 real-time PCR system (Applied Biosystems). All expressions were normalized by GAPDH.

2.3. Immunoblot assay

Total cell lysate was collected and electrophoresed on a 4–12% bis-acrylamide gel at 100 V for two hours. The proteins were transfer to a nitrocellulose membrane with a 300 mA current for 90 min. The primary antibodies used for Western blot were LC3B (Sigma, L7543), mTOR (CST, #4517), p-mTOR (CST, #5536), p-ULK1 (S555) (CST, #5869), pan-Akt (CST, #4685), p-Akt (S473) (CST, #4060), PTEN (CST, #9188), p-PTEN (CST, #9549), p-AMPK (CST, #4188) and GAPDH (CST, #5174).

2.4. Cell proliferation assay

HCT-116 cells were seeded in 96-well plates with 2000 cells per well and treated with a series of peiminine (Chinese National Institute for Food and Drug Control, NIFDC) solutions with final concentrations of 0, 100, 200 or 400 μM. A CCK-8 cell proliferation assay was performed according to the manufacturer's protocol (Dojindo).

2.5. Confocal microscopy

GFP-LC3B stable expression HeLa cells were seeded and cultured in a flask overnight. A gradient concentration of peiminine was added to the cells with final concentrations of 0, 50, 100 and 200 μM. Cells were observed with an Olympus FV1000 (Olympus) confocal microscope 24 h after treatment.

2.6. Flow cytometry

Cells were treated with serial concentrations of peiminine and DMSO solvent, as the negative control, and then cultured in DMEM supplied with 10% FBS for 24 h. The cells were then collected and stained with propidium iodide and Annexin V according to the manufacturer's guidelines (BD Biosciences) and analyzed using a BD Influx™ (BD Biosciences) flow cytometer.

2.7. Immunohistochemistry

Tumors were isolated from xenograft mice after euthanasia and fixed in 5% paraformaldehyde at room temperature for 48 h. Selected samples were embedded in paraffin and sectioned; they were then stained with hematoxylin and eosin; and exposed to cleaved caspase 3 (Biosynthesis Biotech, bs-0087R) and LC3B (Sigma, L7543). The primary antibody LC3B and cleaved caspase 3 were used at the ratio of 1:100. The sections were then mounted for histological analysis.

2.8. Animal experiments

Four-week old female BALB/c nude mice were purchased from Guangdong Experimental Animal Center (Guangzhou, China) and kept in a pathogen-free environment. Approximately 1×10^6 HCT-116 cells were underarm injected into each mouse. One week post tumor implantation, all mice were randomly divided into two groups ($n = 5$): the vehicle (control) group received intraperitoneal (i.p.) injections of saline every two days while the peiminine group received i.p. injections of 3 mg/kg peiminine every two days. At the end of the experiment (two weeks after tumor implantation), all mice were euthanized, tumors were isolated and the weight of each animal was recorded.

2.9. Statistical analysis

All results were expressed as means ± SD from at least three independent experiments. A Student two-tailed *t* test was applied for statistical analysis.

3. Results

3.1. Peiminine induces autophagy in HCT-116 cells

To investigate the autophagy inducing ability of natural compounds derived from Traditional Chinese Medicine, several natural products, including polydatin, peiminine, forsythin and macranthoidin B, were screened in a previous study by assessing the autophagy activation potential in GFP-LC3 stable expression HeLa cells. By comparing the GFP puncta number in drug administration cells and the control, we discovered peiminine was the best autophagy activator among these candidates (Figure S1). Therefore, we applied a gradient dosage of peiminine (50, 100 and 200 μM) in HeLa-GFP-LC3 cells with a control solvent and observed an increase in the GFP puncta in a dose-dependent manner (Fig. 1A). We quantified the GFP-LC3B positive cells according to puncta number per cell, and categorized the cells into three classes (cells with less than 10 puncta per cell, 10–30 puncta per cell and more than 30 puncta per cell). The results showed that under the 50 μM peiminine treatment, the proportion of cells that had 10 or more puncta per cell was significantly elevated (Fig. 1C). To investigate the autophagy inducing capability of peiminine in colorectal

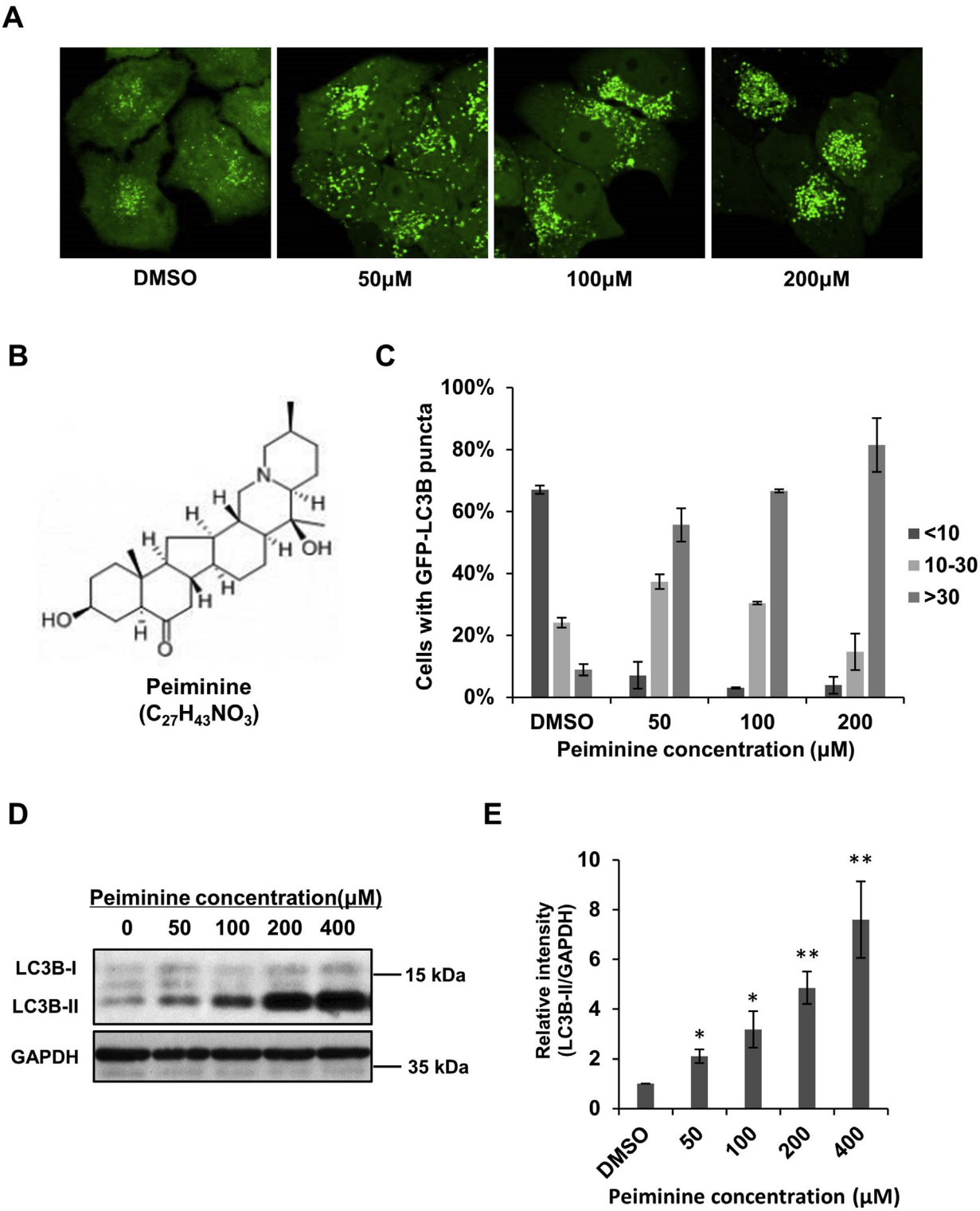

Fig. 1. Peiminine induces autophagy. (A) Accumulation of GFP-LC3B puncta in HeLa cells treated with 0, 50, 100 and 200 μM of peiminine. The distribution of GFP-LC3B was record by confocal microscopy. (B) Molecular structure and formula of peiminine. (C) Quantification of GFP-LC3B puncta by analysis of GFP dots and categorization according to puncta number. Data shown are mean ± SD for >100 independent cells. (D) Dose-dependent increases in LC3B-II/LC3B-I ratio with peiminine treatment at 0, 50, 100, 200 and 400 μM concentrations in HCT-116 cells. (E) Three independent immunoblot assays were performed and gray scale images of each assay were scanned and presented as a bar chart in which the vertical axis is the mean gray scale ± SD. * indicates statistical differences at $p < 0.05$ and ** indicates statistical differences at $p < 0.01$.

carcinoma cells, we applied a gradient concentration (0, 50, 100, 200 and 400 μM) of peiminine to cultured HCT-116 cells and collected the cell lysate for immunoblot assays against LC3B. The result showed a significant elevation of the LC3B-II/LC3B-I protein in the 50, 100, 200 and 400 μM peiminine treated samples, which suggested peiminine induced autophagy in HCT-116 cells (Fig. 1D and E). In addition, in HeLa and MEF cells an increased ratio of LC3B-II/I was observed (Figure S2A and S2B).

3.2. Peiminine enhances autophagic flux by dephosphorylating mTOR through PI3K/Akt and AMPK pathway

Kinase mTOR is a critical regulator of autophagy initiation by forming mTORC1 complexes with protein components, such as raptor and LST8, etc. [22,23]. Phosphorylation of mTOR protein results in the de-phosphorylation of ULK1 (Unc51-like kinase 1), a serine/threonine kinase essential for autophagy initiation. Consequently, this negatively regulates autophagy and/or autophagic flux [24,25]. Therefore, we monitored the protein levels of mTOR and p-mTOR by immunoblot assay. The results showed that peiminine significantly down-regulated the p-mTOR concentration gradient, and there was no change observed in the total mTOR protein level (Fig. 2A). The immunoblot assay indicated that p-ULK1 (S555) was up-regulated upon treatment with peiminine, which suggested an increasing level of autophagic initiation (Fig. 2A). Therefore, our data demonstrated that the autophagic flux was enhanced by peiminine treatment through repressing mTOR phosphorylation and subsequently promoting phosphorylation of ULK1.

To investigate the gene regulation upstream of mTOR, we performed immunoblot assays against proteins, including Akt, PTEN and AMPK, with the phosphorylation forms of these proteins. Post peiminine treated cells were observed to have decreasing levels of p-Akt (S473), but no changes in the pan-Akt levels were observed (Fig. 2A). PTEN was reported to have a negative regulatory effect on the PI3K/Akt signal pathway by transforming PIP_3 to PIP_2 [26]. Different from many other kinases, the phosphorylation of PTEN represses enzyme activity and promotes the production of PIP_3, which consequently up-regulates the down-stream Akt phosphorylation level. We determined the protein levels of PTEN and p-PTEN by immunoblot assays, and the results indicated that p-PTEN was decreased upon treatment with the gradient concentration of peiminine from 0 to 400 μM, while the full PTEN protein level remained steady (Fig. 2A). Our data suggested that the peiminine treatment resulted in the de-phosphorylation of PTEN, which enhanced the inhibitory effects on the PI3K/Akt pathway by de-repressing the PTEN activity.

Another important upstream regulator of mTOR, which is sensitive to energy alteration, is AMPK [27]. We applied immunoblot assays and revealed a significant elevation in the p-AMPK level post peiminine treatment in colorectal carcinoma HCT-116 cells (Fig. 2A). We also investigated an alternative signal pathway that regulates mTOR, and no remarkable differences were observed with or without peiminine treatment (data not shown). A summary of the signal pathway for repressing mTOR phosphorylation post peiminine treatment in colorectal carcinoma cells is expressed in Figure S3A.

Bafilomycin A1 (BAFA1) is a vacuolar-type hydrogen-ATPase inhibitor that increased the amount of LC3B-II by blocking the autophagosome-lysosome fusion [28]. The effect of peiminine on autophagic flux was investigated by exposing cells to BAFA1 (10 nM) for two hours. The result revealed that peiminine-induced

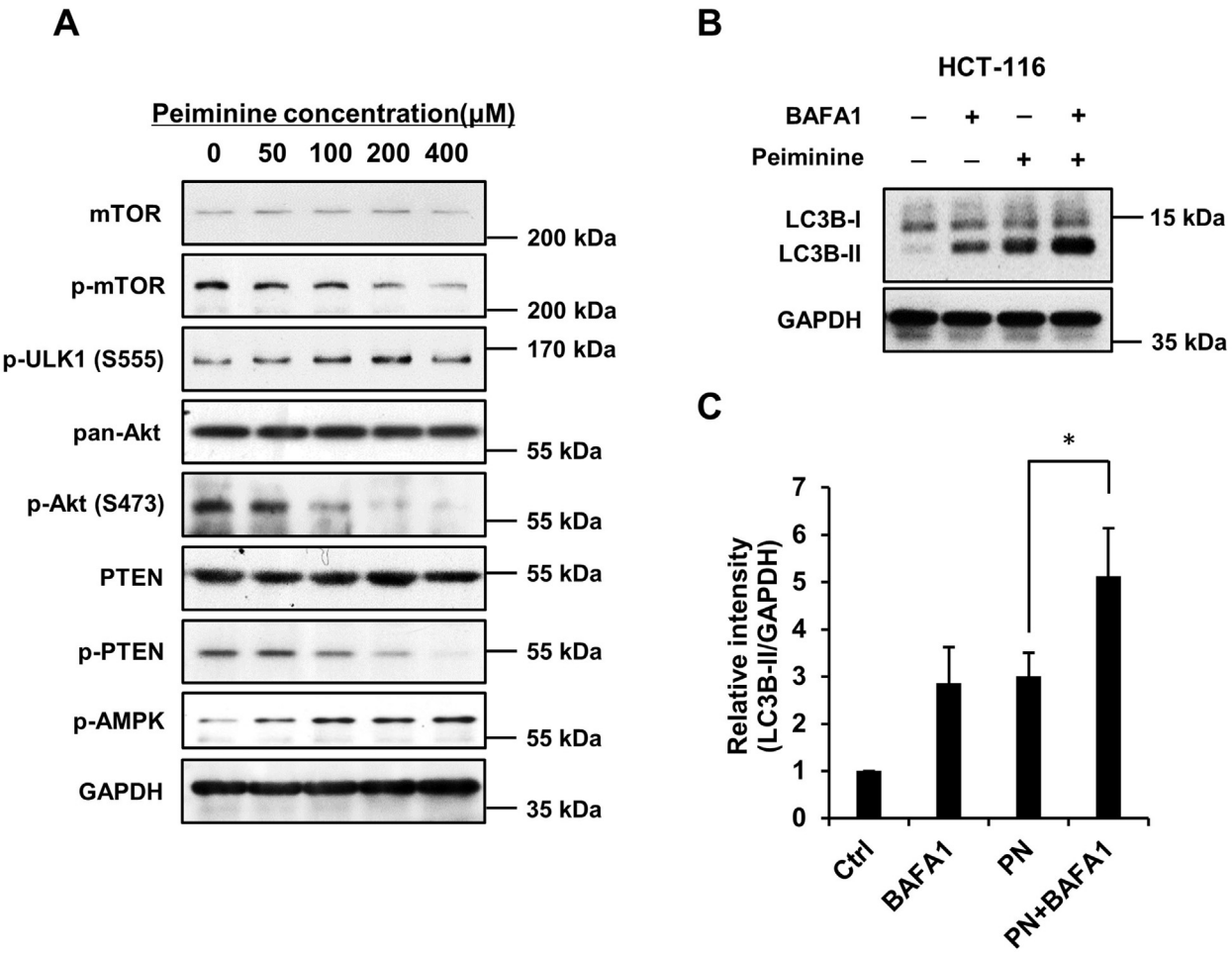

Fig. 2. Peiminine enhances autophagic flux. (A) Immunoblot assay of autophagy regulatory proteins with gradient concentration of peiminine treatment in HCT-116 cells including mTOR, p-mTOR, p-ULK1 (S555), pan-Akt, p-Akt (S473), PTEN, p-PTEN, p-AMPK and internal control GAPDH. (B) Assay of autophagic flux in HCT-116 peiminine treated cells that were supplied with BAFA1. Immunoblot assay performed for LC3B and GAPDH. (C) Gray scale LC3B-II level with 10 nM BAFA1 treatment for two hours.

accumulation of LC3B-II was significantly enhanced by the presence of BAFA1 (Fig. 2B). Gray scale images of Western blot bands were scanned from three independent experiments and summarized as a bar chart that shows BAFA1 significantly elevated the LC3B-II level for 24 h after peiminine treatment (Fig. 2C). As a whole, these results showed that a peiminine treatment could enhance autophagic flux in HCT-116 cells.

3.3. *Peiminine induces autophagic cell death in HCT-116 cells*

In a previous study, we discovered that treating colorectal carcinoma HCT-116 cells with peiminine resulted in a decrease in cell viability in a dose-dependent manner (Figure S2C). We performed flow cytometry to quantify the apoptotic cell death after treatment with peiminine (0, 100, 200 and 400 μM) in DMEM culture medium using the Annexin V apoptosis assay. The results showed that peiminine induced cell death depending on the drug concentration (Fig. 3A). Three independent assays were performed and the percentage cell death for each concentration gradient was determined, which indicated a significant increase in the Annexin V positive cells in those treated with peiminine at 200 and 400 μM (Fig. 3B). In addition, it was shown that the 200 and 400 μM peiminine treatments also induced apparent early apoptosis (Fig. 3B). To further validate the apoptosis inducing effects of peiminine, immunoblot assays of caspase 9 and cleaved caspase 3 were performed for HCT-116 cells treated with 400 μM peiminine for 24 h. The result showed

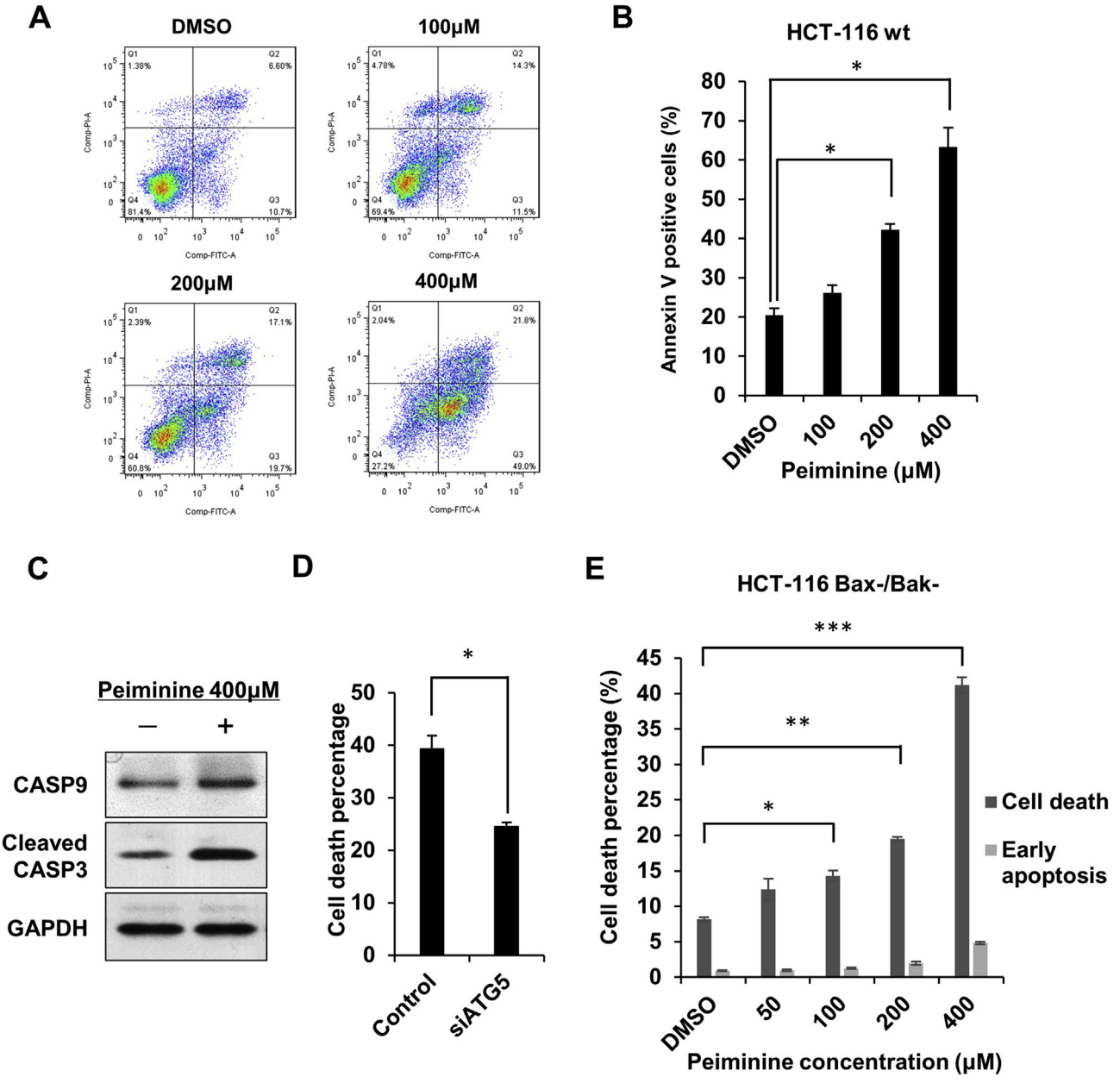

Fig. 3. Peiminine induces autophagic cell death. (A) Double staining of PI and Annexin V for flow cytometry of gradient concentration peiminine treated HCT-116 cells with vehicle control (DMSO) for 24 h. (B) Percentage of Annexin V positive cells calculated from three independent experiments for gradient treatment with peiminine. * indicates statistical differences at $p < 0.05$. (C) Immunoblot of caspase 9 (CASP9) and cleaved caspase 3 (CASP3) with or without peiminine treatment. (D) Cell death percentage in HCT-116 cells with peiminine treatment after transfection of ATG5 siRNA and randomized sequence control. * refers to statistical differences at $p < 0.05$. (E) Cell death of Bax-/Bak- DKO HCT-116 cells determined by flow cytometry after double staining for PI and Annexin V 24 h post treatment with gradient concentration of peiminine. * indicates statistical differences at $p < 0.05$, ** indicates statistical differences at $p < 0.01$ and *** indicates statistical differences at $p < 0.001$.

that the apoptotic signatures were all elevated post peiminine treatment, which confirmed the apoptosis enhancement effect (Fig. 3C).

Since peiminine induces significant autophagy and promotes apoptosis in colorectal carcinoma cells, we were curious as to how these two paths of programmed cell death, apoptosis and autophagy, contribute to the cell death induced by peiminine treatment. Firstly, we designed and synthesized a set of siRNA duplexes against ATG5, an E3 ubiquitin ligase, which is essential for autophagosome elongation. The down-regulation or knock-out of ATG5 directly compromises the autophagy process. We validated the efficiency of ATG5 siRNA by immunoblot assay and the results showed a clear reduction in the ATG5 protein level post ATG5 siRNA transfection when compared with the non-specific sequence of the small RNA control (NC) (Figure S2D). Therefore, we transfected ATG5 siRNA with the control small RNA duplex into wild type HCT-116 cells and treated them with peiminine at 200 μM for 24 h. Cells were collected to perform flow cytometry to monitor cell death. We discovered that the peiminine-induced cell death was greatly decreased after the ATG5 siRNA treatment, which indicates that autophagy plays an important role in peiminine-induced cell death (Fig. 3D).

To further investigate the cell death induced by autophagy, we used HCT-116 Bax/Bak-deficient cells to assess the effect of peiminine in apoptosis-null cells. We treated HCT-116 Bax/Bak-deficient cells with 100, 200 and 400 μM of peiminine and DMSO (solvent) as a control for 24 h. Cells were double stained to identify PI and Annexin V and then harvested for flow cytometry. The result showed that peiminine induced prominent cell death in the Bax-/Bak-cells from the 100 μM concentration, and that 400 μM of

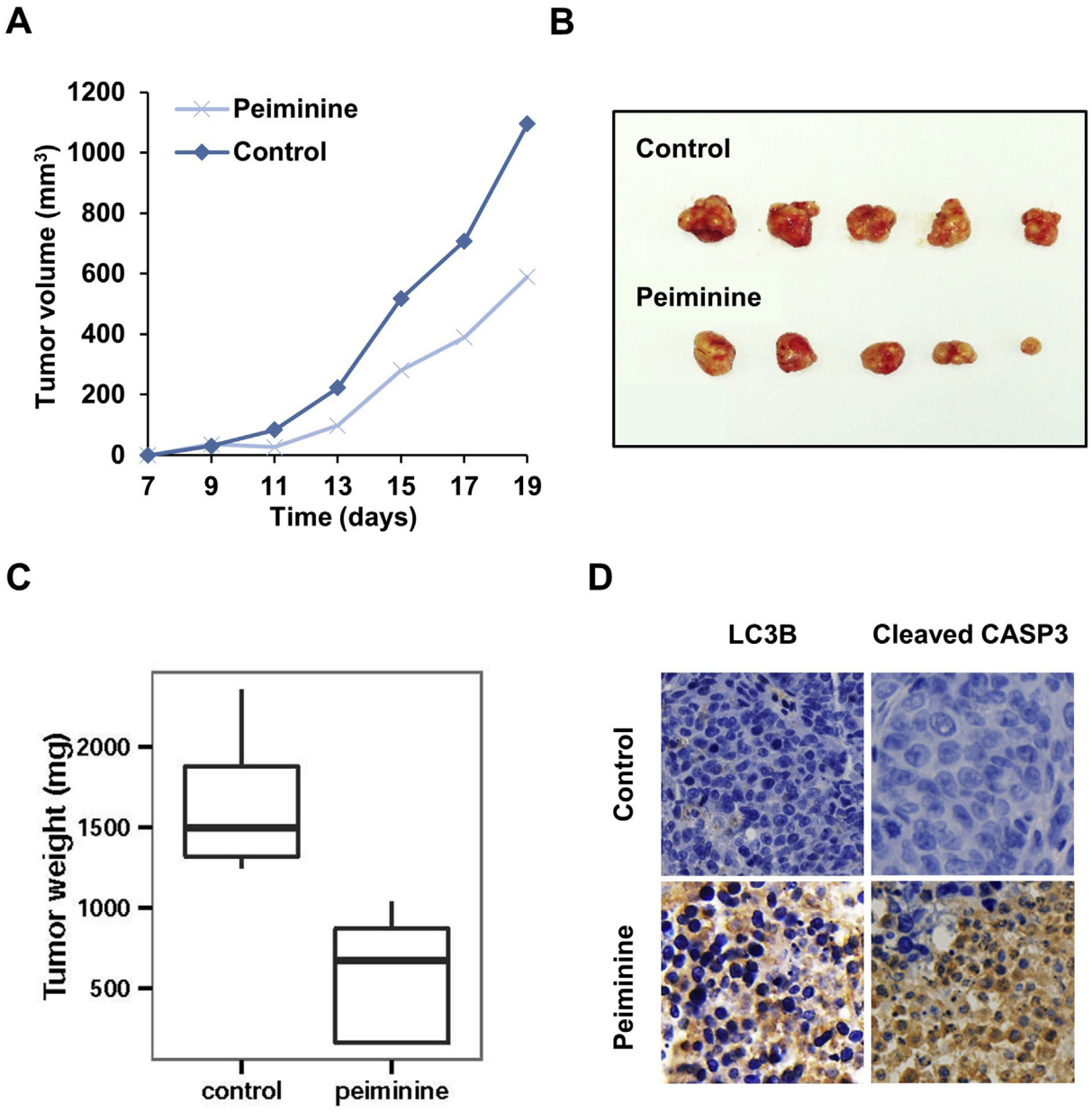

Fig. 4. Peiminine represses colorectal carcinoma xenograft tumor growth *in vivo*. (A) Four-week old female nude mice were engrafted with HCT-116 cells (1×10^6 cells) and randomly divided into two groups. Tumor-bearing mice were treated with the vehicle (control) and peiminine (3 mg/kg) by intraperitoneal injection every two days for a total of three weeks. Tumor volumes were measured and calculated every two days. (B) Images of tumors from (A). (C) Tumor weight from (B). (D) Immunohistochemical staining for LC3B and cleaved caspase 3 (CASP3).

peiminine resulted in 40% cell death in the apoptosis-null cells, while only a few early-apoptosis cells were detected ($p < 0.001$) (Fig. 3E and S2B). This result demonstrated that peiminine induces autophagic cell death as well as apoptosis in colorectal carcinoma cells.

3.4. Peiminine represses xenograft tumor growth in vivo

To investigate the antitumor activity of peiminine *in vivo*, four-week old female nude mice were injected with human colorectal carcinoma cells, HCT-116, and then administer with peiminine or the vehicle control through i.p. injections. The i.p. injections were administered one week post carcinoma cell injection, and the tumor volume was monitored every two days. We discovered that tumor volume dramatically increased in the vehicle group (control), while in the peiminine administration group (peiminine) it was significantly less prominent (Fig. 4A). Two weeks post tumor cell injection, mice were euthanized and tumor tissues were removed and weighted. As shown in Fig. 4B and C, the peiminine treatment significantly repressed the tumor growth compared to the control.

Finally, immunohistochemistry of the tumor tissues was performed to determine the levels of LC3B and cleaved caspase 3. Little LC3B staining was observed in the control mice, while peiminine treated mice exhibited a significantly increased level of LC3B staining. We also observed a significant increase in the staining of the cleaved caspase 3, which suggested that peiminine induced cell death related to apoptosis, at least partially (Fig. 4D). Together, these results indicate that peiminine-mediated inhibition of tumor growth is associated with apoptosis and autophagic cell death.

4. Discussion

Autophagy is sustained at low physiological levels in cells to maintain homeostasis of the cell's inner environment by regulating protein and organelle turnover. It is dramatically upregulated when starvation or growth factor withdrawal occurs, and then it generates “recycled” intracellular nutrients and energy. Autophagy is also enhanced in many other physiological situations, including adipogenesis (removal of late stage mitochondria) and pathological processes, such as oxidative stress and infection. Kinase mTOR is the most important regulator that inhibits autophagy under the normal condition. De-phosphorylation of mTOR was controlled by upstream signals, e.g. PI3K/Akt, AMPK and p53, etc., which subsequently de-represses the down-stream ULK1 and initiates the autophagy process. Following treatment with peiminine it was observed that the level of phosphorylation of mTOR kinase was down-regulated. In addition, tracking back upstream from mTOR demonstrated that two signal pathways were involved, PI3K/Akt and AMPK. An increasing level of p-AMPK was identified, which may result in the repression of p-mTOR and the elevation of p-ULK1, both of which activated autophagy flux upstream. Decreasing p-Akt (S473) compromised the function of mTOR kinase, and we revealed that de-repression of PTEN contributed to the inhibition of the PI3K/Akt pathway. PI3 kinase type I is activated by growth factors such as insulin, and future studies should concentrate on revealing the peiminine function site in this pathway. In addition, the investigation of the regulatory mechanism of phosphorylation of PTEN and AMPK is another way to proceed.

The concept of programmed cell death has been considered for many years, and much evidence has revealed the phenomenon of autophagic cell death. Activation of autophagy is generally associated with cell survival under nutrient depletion, etc., by “transforming” cellular contents into reusable energy [29]. However, it is conceivable that excessive autophagy could eventually destroy a cell by “removing” critical organelles and molecules for cell survival. Bax/Bak-deficient mouse embryonic fibroblast cells undergo non-apoptosis after exposure to etoposide or staurosporine [20]. Subcellular observation under an electron microscopy revealed that drug-treated Bax/Bak-deficient cells contained many double-membrane vesicles, which were finally demonstrated to be autophagosomes. Further experiments demonstrated that the knockdown of Beclin-1 greatly reduced cell death, which confirmed the direct association of autophagy and cell death. Therefore, autophagic cell death can be understood as cell death induced by uncontrolled autophagy, which features cell death suppressed by the inhibition of autophagy by chemical inhibitors or genetic knockdown.

The identification of natural compounds that either induce or repress autophagy is vital for identifying therapies for cancer or other diseases. In this study, we revealed the autophagy-inducing effect of peiminine in colorectal carcinoma cells. We also demonstrated that peiminine enhances autophagic flux by repressing mTOR function through influencing the phosphorylation level of Akt and AMPK. When the expression of ATG5 was knocked down, peiminine-induced cell death decreased, which suggests that autophagy plays a critical role in this process. Furthermore, we treated non-apoptotic Bax/Bak-deficient HCT-116 cells with peiminine and the results showed increasing cell death as the concentration of peiminine increased. In summary, our study indicated that peiminine induces autophagic cell death by enhancing autophagic flux through repressing mTOR activity via multiple aspects, which has broad therapeutical prospects in cancer treatment.

Conflict of interest

None.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.102.

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