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Research Article

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The peiminine stimulating autophagy in human colorectal carcinoma cells via AMPK pathway by SQSTM1

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Abstract: Autophagy is a conserved catabolic process, which functions in maintenance of cellular homeostasis in eukaryotic cells. The self-eating process engulfs cellular long-lived proteins and organelles with double-membrane vesicles, and forms a so-called autophagosome. Degradation of contents via fusion with lysosome provides recycled building blocks for synthesis of new molecules during stress, e.g. starvation. Peiminine is a steroidal alkaloid extracted from *Fritillaria thunbergii* which is widely used in Traditional Chinese Medicine. Previously, peiminine has been identified to induce autophagy in human colorectal carcinoma cells. In this study, we further investigated whether peiminine could induce autophagic cell death *via* activating autophagy-related signaling pathway AMPK-mTOR-ULK by promoting SQSTM1(P62). Xenograft tumor growth *in vivo* suggested that both peiminine and starvation inhibit the growth of tumor size and weight, which was prominently enhanced when peiminine and starvation combined. The therapeutical effect of peiminine in cancer treatment is to be expected.

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1 Introduction

Autophagy is an evolutionarily conserved cellular pathway that delivers cellular contents to lysosomes for degradation. Three types of autophagy have been identified as chaperone-mediated autophagy, microautophagy and macroautophagy [1]. Autophagy is an important regulatory process in eukaryotic cells for removing long-lived molecules and organelles; during autophagy, autophagosomes are formed, engulfed with cytoplasm and organelles, and followed by fusion with lysosomes for degradation. The degraded products were recycled for new biomolecule synthesis [2]. Autophagy is involved in numerous physiological and pathological processes, such as starvation and tumorigenesis etc. The role of autophagy in tumorigenesis is complicated. On one hand, autophagy provides "recycled" energy within cells and benefits to cell growth and proliferation under biological stress; on the other hand , excessive autophagy exhausts the cell contents, which results in cell death. However, in cancer cells, the autophagy-related protein levels are lower than in normal cells; failure of autophagy would end up in tumorigenesis [3].

Autophagic cell death, also named as type II cell death, is an alternative pathway of programmed cell death. In those cells lacking Bax and Bak, proapoptoic members of Bcl-2 family essential for apoptosis process, cells do not undergo apoptosis in exposure of stimuli [4]. Alternative pathways were replaced which were responsible for cell death in these cells. Moreover, the engined GFP-LC3 cellular system is helpful to provide the formation of autophagosomes which is implicated in autophagic cell death.

Peiminine, one of the main effective components of *Fritillaria thunbergii*, is widely used for treatment of various diseases including cancer [5,6]. However, few studies have elucidated the biological function of peiminine *in vitro* and *in vivo*.

Previously, we investigated whether administration of peiminine in colorectal carcinoma cell HCT-116 induced phenomenal activation of autophagy, indicated by accumulation of GFP-LC3B puncta, as well as being confirmed in Bax-/Bak- HCT-116 cells. It was highlighted that significant tumor growth has a repressive effect of peiminine *in vivo* [7]. To further understand the mechanism of peiminine-induced autophagy in colorectal carcinoma cells, SQTEM1, which localized at the autophagosome formation site and interacted with LC3, was monitored. PTEN and AMPK/mTORC/ULK signaling pathway were determined when cells were treated with peiminine. In addition, xenograft tumor growth *in vivo* was performed to observe the therapeutic effect of peiminine, in combination with starvation.

2 Materials and methods

2.1 Cell culture and transfection

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

2.2 RNA isolation and quantitative PCR analysis

Total RNA was isolated with Trizol (Invitrogen, USA) and purified using RNAeasy (Qiagen, CA) following the manufacturers protocol. First strand cDNA was reversely transcripted using random primer and M-MLV (Takara). Quantitative real-time PCR was performed using SYBR Master Mix (TOYOBO) with specific primers on AB7300 real-time PCR system (Applied Biosystems). All objective gene expression was normalized by endogenous GAPDH.

2.3 Microarray expression profiling analysis

PrimeView™ Human Gene Expression Array was used for gene expression profiling of cell lines. Three biological replicates were analyzed for the vehicle control group and peiminine group. Statistical analysis of expression patterns were performed with AGCC software (Affymetrix® GeneChip® Command Console® Software). Probes between 20-100 percentile were filtered; *t*-test with FDR multiple testing correction was used and a filter of 2-fold change was used to identify up-regulated or down-regulated probes. The criterion used for significantly dysregulated genes was $p < 0.05$ and a 2-fold or higher change. Hierarchical cluster analysis was performed. In addition, the gene list was further analyzed for function and pathway analysis.

2.4 Western blot

Total cell lysates were collected and electrophoresis was performed in 4-12% bis-acrylamide gel with 100V for 2 hours. Proteins were transfered to nitrocellulose membrane with 300 mA current for 90 minutes. Primary antibodies used for Western blot included 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).

2.5 Animal experiments

Four-week old female BALB/c nude mice were purchased from Guangdong Experimental Animal Center and were kept in a pathogen-free environment. Approximately 1×10^6 HCT-116 cells were injected subcutaneously into each mouse in the right axillary region. One-week post tumor cell implantation, all mice were randomly divided into four groups: vehicle control group, peiminine group, starvation group and peiminine + starvation group (n = 5 in each group). The peiminine group received i.p. injection of peiminine at dosage of 3 mg/kg weight every two days. The starvation group was fed with 50% normal diet daily. The peiminine + starvation group received i.p. injection of peiminine at dosage of 3 mg/kg weight every two days and fed with 50% normal diet daily. Three weeks after tumor implantation, all mice were sacrificed under anaesthesia, and tumors were isolated and weighted separately.

2.6 Immunohistochemistry

Tumors were isolated from xenograft mice and fixed in 5% paraformaldehyde in paraffin and sectioned. Then the

samples were stained with hematoxylin and eosin, as well as cleaved Caspase 3 (Biosynthesis Biotech, BS-0087R) and LC3B (Sigma, L7543). The primary antibodies were used at the ratio of 1:100. The sections were then mounted for histological analysis microscopically.

2.7 Statistical analysis

All results were expressed as mean ± SD from at least three independent experiments. A student two-tailed *t* test was applied for statistical analysis using SPSS Ver.19 for Windows (SPSS Inc., USA). Significant difference was indicated by $p < 0.05$.

3 Results

3.1 Peiminine induces autophagy-related protein expression in HCT-116 cells

First, gene array was applied to indicate autophagy-related change in HCT-116 cells after peiminine administration. Among genes with significant change, YY1, an epigenetic regulator, was observed to be up-regulated by peiminine significantly (Fig.1A).

Expression of SQSTM1, which is a target of YY1, was determined by quantitative PCR. In peiminine-treated HCT-116 cells, mRNA levels of YY1 and SQSTM1 were significantly higher than in the control group (Fig.1B).

Then, significant increases in SQSTM1 and LC3B-II/LC3B-I protein expressions were observed following administration to 50, 100, 200 and 400 μM peiminine in HCT-116 cells. In addition, time-course change of SQSTM1 and LC3B expression were also performed, which suggested autophagy flux reflected within 4 h post peiminine treatment (Fig.2B).

Bafilomycin A1 (BAFA1) is a vacuolar-type hydrogen-ATPase inhibitor which remarkably increased the amount of LC3B-II and SQSTM1 by blocking autophagosome-lysosome fusion. The result revealed that peiminine-induced accumulation of LC3B-II and SQSTM1 were significantly enhanced in the presence of BAFA1 (Fig.2C and D).

Together, these data indicated that in peiminine-treated HCT-116 cells, the expression of autophagy-related markers was induced.

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

To further explore the mechanism of peiminine induced LC3B accumulation, the PTEN and AMPK/ mTORC/ULK signaling pathway was tracked.

When HCT-116 cells were treated with AMPK inhibitor Dorsomorphin, the protein level of AMPK decreased prominently, followed by a down-regulation of SQSTM1 and LC3B I/II (Fig.3B). In addition, in a PTEN siRNA transfected HCT-116 cell model, decrease in PTEN expression, followed by increase in SQSTM1 and LC3B I/II were observed (Fig.3C), as compared to that in the control group.

When HCT-116 cells were treated with varying concentrations of peiminine, significantly down-regulated p-mTOR in a dose-dependent manner was seen, but no alternation was observed in total mTOR protein level (Fig.3D). The p-ULK1 (S555) was up-regulated upon

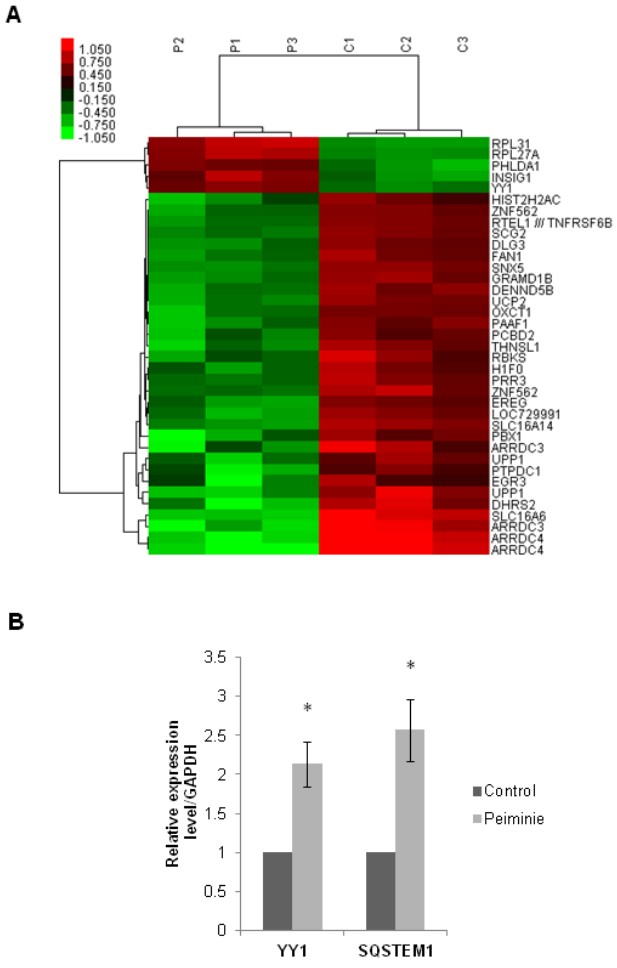
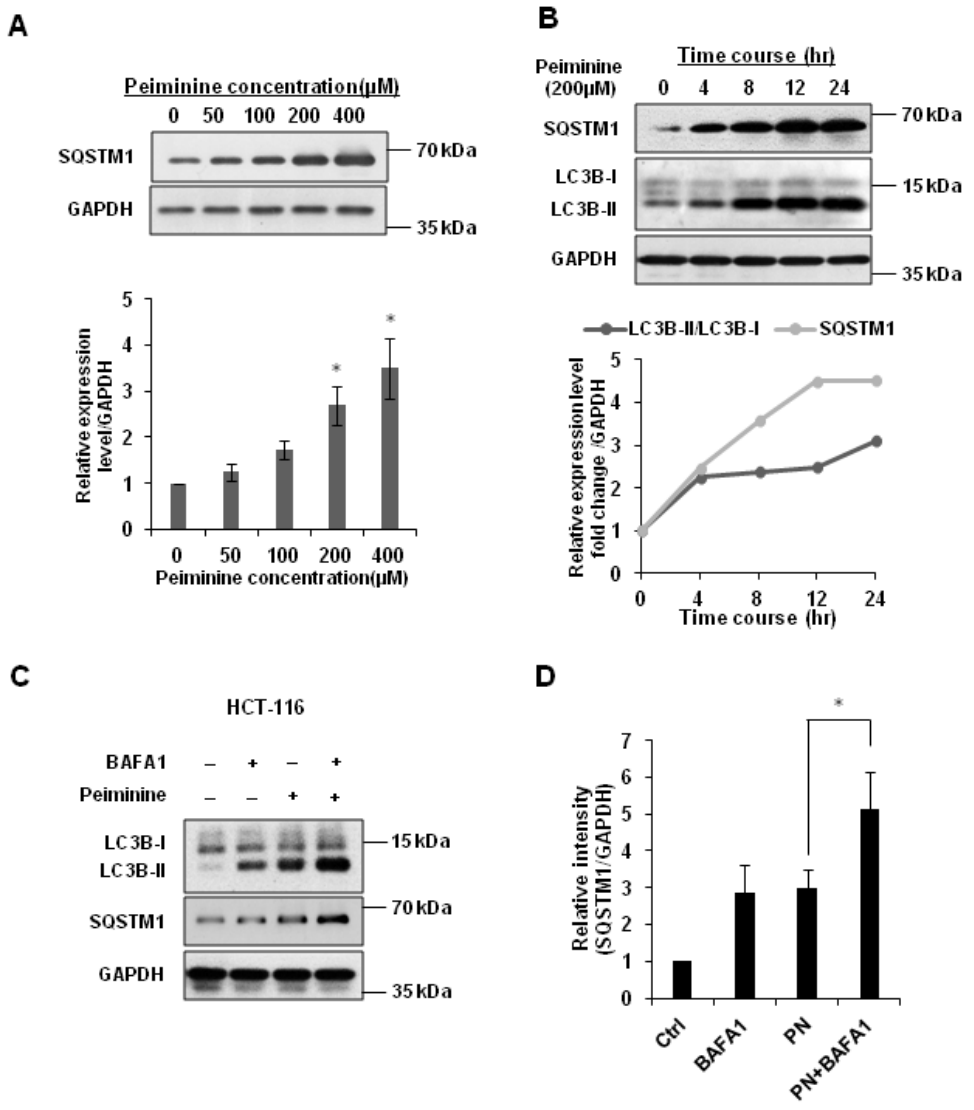


Figure 1. (A) clustering analysis of gene expression and (B) mRNA level of YY1 an SQSTM1 after peiminine treatment in HCT-116 cells.



Figue 2. Peiminine enhances autophagic flux. (A) Western blot result of autophagy regulatory protein of SQSTM1 with increased concentration of peiminine treatment in HCT-116 cells. (B) Time-dependent expression of autophagy regulatory proteins SQSTM1 and LC3B-II/LC3B-I ratio in 200μM peiminine-treated HCT-116 cells. (C) Assay of autophagic flux in peiminine-treated HCT-116 with supplement of 10nM BAFA1. (D) LC3B-II level in HCT-116 cell with 10nM BAFA1 treatment for two hours.

treatment of peiminine which suggested an increasing level of autophagic initiation (Fig.3D).

Numerous signal pathways were validated to have regulatory effects on mTOR protein function including AMPK, phosphatidylinositol 3-kinase (PI3K)/Akt pathways etc. To investigate the gene regulation upstream of mTOR, we determined protein levels including Akt, PTEN and AMPK with phosphorylation forms of these proteins. Decrease in p-Akt (S473) protein level was firstly observed post peiminine treated cells and no significant change was detected in pan-Akt level, which suggested activation of PI3K/Akt signal pathway (Figure 3D). The p-PTEN was remarkably decreased upon administration to varying concentrations of peiminine, while full PTEN protein level remained steady. It suggested peiminine induced de-phosphorylation of PTEN, which inhibited PI3K/Akt pathway by de-repressing PTEN activity. In contrast, significant elevation of p-AMPK level post peiminine treatment in colorectal carcinoma cell HCT-116 was observed (Fig.3D).

Therefore, our data demonstrated that autophagic flux was enhanced by peiminine treatment through repressing mTOR phosphorylation and subsequently promoting phosphorylation of ULK1; peiminine enhanced p-AMPK and decreased p-PTEN molecularly, which may explain the increase in SQSTM1 and LC3B I/II.

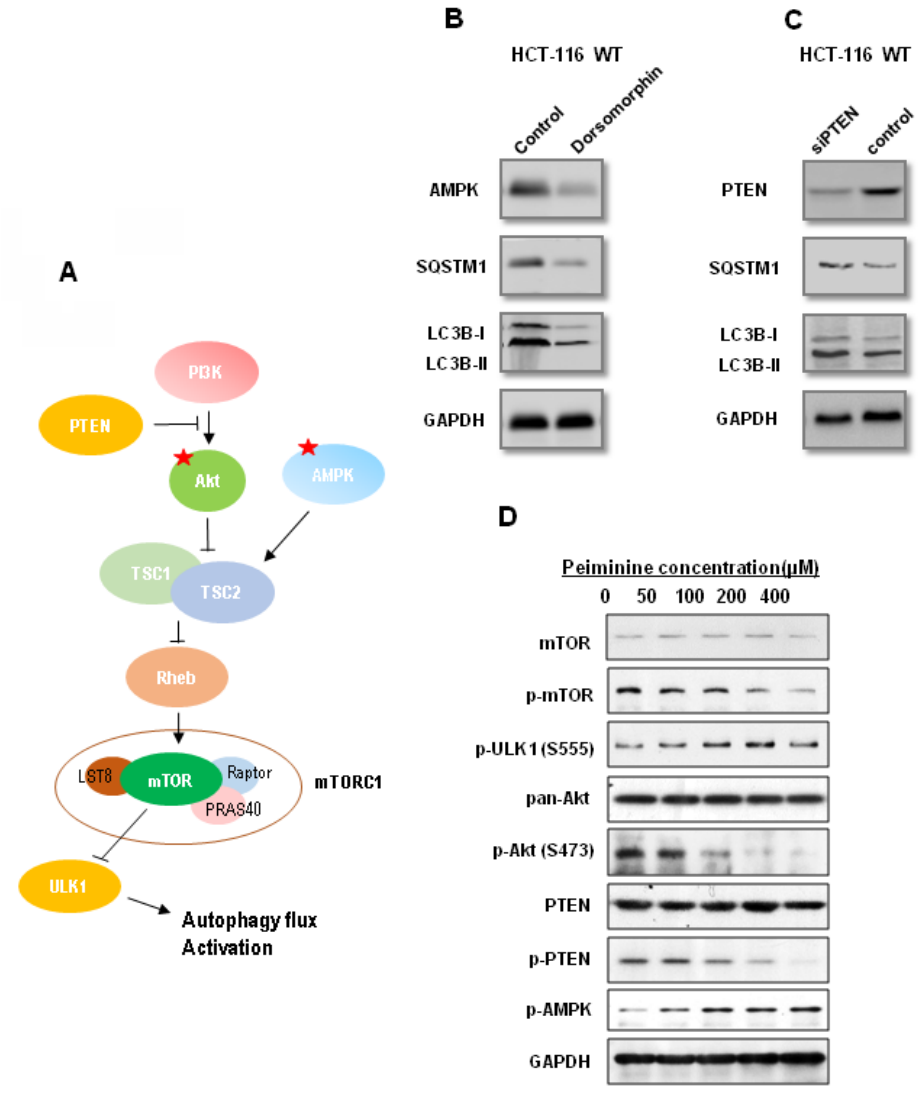


Figure 3. Peiminine represses mTORC1 function by repressing Akt phosphorylation and activating AMPK phosphorylation. (A) Schematic demonstration of AMPK/mTORC/ULK1 signaling pathway. (B) Western blotting result of SQSTM1 and LC3B expression post PTEN siRNA in HCT-116 cells. (C) Western blotting result of SQSTM1 and LC3B expression post AMPK inhibitor Dorsomorphin in HCT-116 cells. (D) Western blotting result of autophagy regulatory proteins including mTOR, p-mTOR, p-ULK1, pan-Akt, p-Akt (S473), PTEN, p-PTEN, p-AMPK and internal control GAPDH.

3.3 Peiminine represses xenograft tumor growth *in vivo*

To investigate the antitumor activity of peiminine *in vivo*, four-week female nude mice were injected with human colorectal carcinoma cells, HCT-116, and then administrated with peiminine or vehicle control through intraperitoneal injection respectively. Tumor volume was measured every 2 days since one-week post-carcinoma cell injection. We discovered that tumor volume was dramatically increased in the vehicle group (Ctrl) whereas the peiminine administration group (peiminine) was significantly less prominent (Fig.4A). Three-week post-tumor cell injection mice were sarcrificed and tumor tissues were removed and weighed. Our data showed tumor size and weight in the peiminine-treated group were significantly less than that in vehicle delivered control group (Fig.4B and C).

Starvation (caloric restriction) is a potent physiological inducer for autophagy. In order to investigate the therapeutic possibility of combined treatment of peiminine and starvation *in vivo*, we fed xenograft mice with 50% of regular dietary and divided mice into starvation plus peiminine (PN+SR) and starvation plus vehicle (Ctrl+SR) to perform the same procedures as the previous experiment. The result showed that the tumor

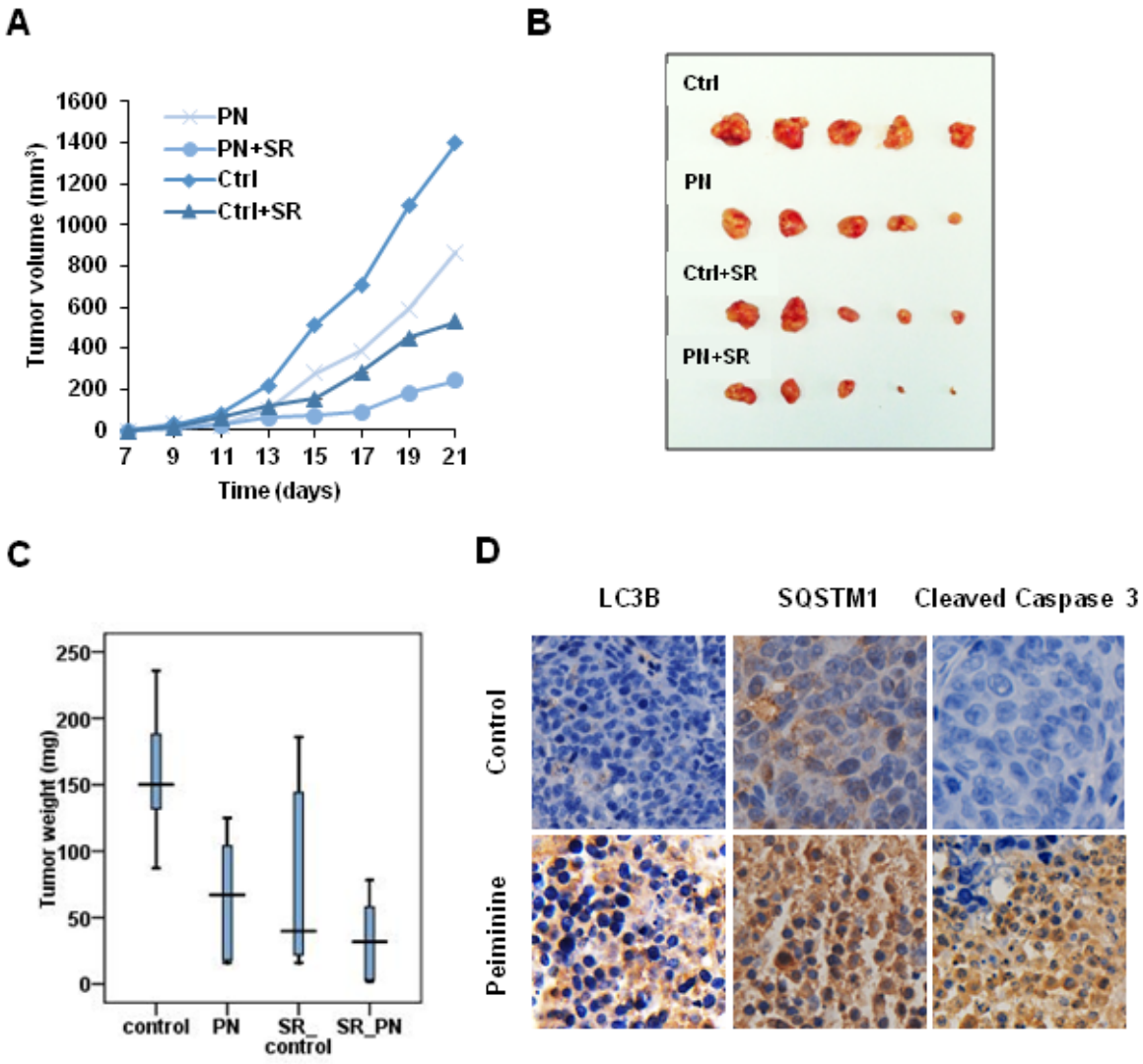


Figure 4. Peiminine represses colorectal carcinoma xenograft tumor growth *in vivo*. (A) Four-week old female nude mice were engrafted with HCT-116 cells (1×10^6 cells) and randomly assigned into four groups: control (ctrl), peiminine (PN), control+starvation (Ctrl+SR) and peiminine+starvation (PN+SR). (A) Measurement of tumor volumes every two days. (B) Images of tumors from HCT-116 cells-injected nude mice. (C) Measurement of tumor weight. (D) Immunohistochemical staiing for LC3B, SQSTM1 and cleaved Caspase 3.

size and weight of starvation-peiminine combination group were significantly lower than other groups (Fig.4).

Finally, immunohistochemistry of tumor tissues were performed to determine the levels of LC3B, SQSTM1 and cleaved Caspase 3. Little LC3B staining was observed in control mice while peiminine treated mice exhibited significantly increased levels of LC3B staining. SQSTM1 expression level in the control group was lower than in the peiminine-treated group (Fig.4D). In addition, a significant increase in positive-staining of the cleaved Caspase 3 was seen (Fig.4D). Taken together, these results indicate that peiminine-mediated tumor growth inhibition is associated with apoptosis and autophagic cell death.

4 Discussion

Previous understanding believed that autophagy assists cells to overcome biological stress as an antagonist of cell death. In one study, administration of peiminine to wild-type HCT-116 cells caused apoptosis; cytochrome c trigged caspase activation indicated the involvement of caspase-dependent cell death. However, when non-apoptotic Bax/Bak-deficient HCT-116 cells were treated with peiminine, increased cell death was observed together with the increase in peiminine concentrations, as compared to the control group [7]. These data highlighted the course of autophagy in presence of peiminine in colorectal carcinoma cells.

YY1, a member of GLI-Kruppel class of zinc finger proteins, plays a significant role in many biological processes; YY1 knockdown inhibited cell viability and autophagy flux through downregulating SQSTM1. SQSTM1, as a substrate in autophagy process, is usually regarded as a reference for autophagic flux [8]. As shown in Fig.1, significant increase in YY1 expression was observed, together with alterations in expression of SQSTM1.

Recent studies observed wide existence of autophagy accompanied with cell death. However, autophagy functions are differences. On one hand, increase in autophagy induces cell death in normal cells; on the other hand, in cancer cells, if cells fail to execute autophagy, the tumor progresses finally [3].

SQSTM1, which is also called p62, is extensively existing in tumor cells. It functions as a receptor for ubiquitinated proteins, organelles and microbes to sequester them into autophagosome [9,10]. It interacts with LC3 and therefore involves in the process of autophagy; the accumulation of SQSTM1 in autophagy-deficient mice illustrates a connection between autophagy and SQSTM1 [11,12]. As a biomarker of autophagy, the conversion of soluble LC3-I to autophagic-vesicle-associated form LC3-II was found in peiminine-treated HCT-116 cells [7]. Autophagy is increased in many physiological situations, and kinase mTOR is one of the crucial regulators that control autophagy [13-15]. In cancer cells, the expression of autophagy related proteins is lower than that in normal cells, if cells fail to execute autophagy, the tumor progresses [16,17]. Previous studies identified that insufficient activation of autophagy-related signaling pathway in cancer cells results in insufficient autophagy, which further induces malignant phenotype [18,19]. Based on the data revealed in Fig.3, the signaling pathway of peiminine-induced elevation in autophagic flux involves repressing phosphorylation of mTOR through inhibiting upstream of PI3K/Akt and AMPK.

PTEN siRNA induced upregulation of SQSTM1 and LC3B II/I; similar result was observed when AMPK inhibitor was applied in HCT-116 cells. In accordance to these data, peiminine boosted the expression of SQSTM1 and LC3B II/I, in a dose-dependent and time-dependent manner (Fig.2 and 3). Taken together, peiminine enhanced autophagy in colorectal carcinoma cells by upregulating the autophagy-related signaling pathway.

In addition, autophagy functions differentially rely on the position it stands. On one hand, it provides "recycled" energy for cells under biological stress condition; on the other hand, excessive autophagy results in uncontrolled degradation of cellular content, which may cause cell death. As a later stage, it is widely known that when autophagosomes fusion with lysosomes, the formation of autolysosomes ensures the degradation and supplement of recycled energy, as in the case of starvation [20]. Activation of autophagy further contributes to the tolerance of nutrient deprivation in colorectal carcinoma [21,22]. Therefore, it is convincible that blockage in the autophagosome-lysosome fusion event inhibits the energy reuse. In our study, LysoTracker Red and GFP-LC3B co-staining suggested that the fusion of autophagosomes and lysosomes is impaired by the administration of peiminine (Fig. S1). Even though the data are limited, they suggested that blockage of autophagosome-lysosome fusion may lead to cell death as a result of poor adaption of colorectal carcinoma cells to peiminine treatment.

The antitumor activity of peiminine was investigated *in vivo*. Both peiminine and starvation inhibit the growth of tumor in size and weight, which was prominently enhanced with peiminine and starvation combined.

In conclusion, our study indicated that peiminine could induce autophagic cell death *via* activating autophay-related signaling pathway of AMPK-mTOR-ULK by promoting SQSTM1 expression. The therapeutical effect of peiminine in cancer treatment is to be expected.

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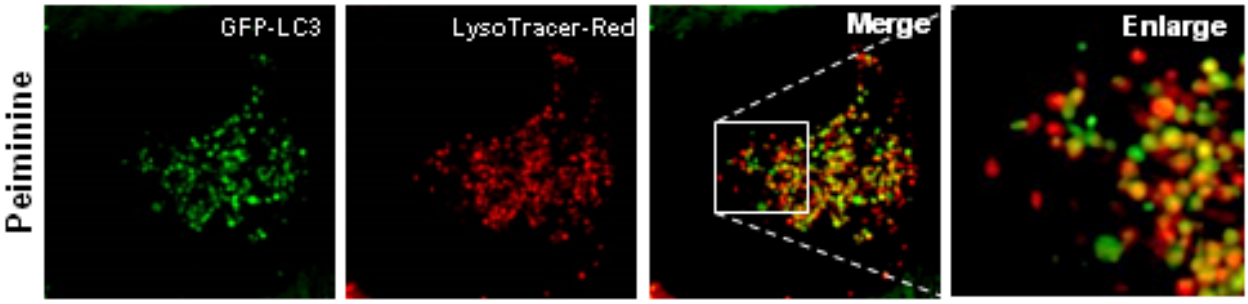

Figure S1. Peiminine blocks autophagosome-lysosome fusion.