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Research

Lysionotin promoted apoptosis of hepatocellular carcinoma cells via inducing autophagy

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Abstract

Background Hepatocellular carcinoma is a prevalent malignant tumor with a high mortality rate. Natural plants hold promise for its treatment, however, the mechanism of lysionotin induced apoptosis in liver cancer cells unclearly. This study aims to investigate the microenvironment alterations and the efficacy of lysionotin in liver cancer.

Methods Transmission electron microscopy, and laser confocal microscopy were employed to investigate the effect of lysionotin on autophagy in HCC cells. The molecular mechanism through which lysionotin induces autophagy and autophagy-induced apoptosis was ascertained by transcriptome sequencing, immunoblotting and Hoechst 33258 staining.

Results RNA sequencing analysis, electron microscopy and laser confocal microscopy revealed that lysionotin initiate autophagy in liver cancer cells. Immunoblotting indicated that lysionotin markedly enhances the activation of LC3-II in HCC cells, resulting in the activation of key effector molecules ATG12, Beclin-1 and the degradation of P62. Combined with autophagy inhibitors CQ and 3-MA significantly inhibited lysionotin-induced cell apoptosis. Immunoblotting and Hoechst staining disclosed that the activation of autophagy by lysionotin might be associated with the suppression of the mTOR-AKT signaling pathway. The treatment of mTOR inhibitor RAPA and activator 1485 demonstrated that inhibiting mTOR activation significantly augments the pro-apoptotic effect of lysionotin on liver cancer cells, while mTOR activator could rescue the effect of lysionotin on cells.

Conclusions The findings suggest that the activation of autophagy by lysionotin may represent one of the pivotal mechanisms underlying its therapeutic efficacy against HCC and its synergistic enhancement of RAPA's antitumor effects.

Keywords Hepatocellular carcinoma (HCC) · Lysionotin · Autophagy

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1 Introduction

Liver cancer is the second most common type of cancer, characterized by rapid progression of malignancies and a poor prognosis [1]. Hepatocellular carcinoma (HCC) is the most common type of the liver cancers, about 90% [2, 3].

Fortunately, thanks to the application of multi-slice spiral CT and the more advanced dual-source CT, data acquisition and image reconstruction technologies have been continuously enhanced, thereby guiding artificial or surgical robots to achieve precise incision of liver lobes and liver segments, which plays an even more significant role in the clinical diagnosis and treatment of liver cancer [4], especially in the early stages of liver cancer, most patients can be treated by surgical resection; Intermediate-stage can be considered transaterial therapies; Advanced disease should be recommended for patients with molecular targeted agents or immune checkpoint inhibitors [5, 6]. Despite the advancements in the systemic treatment of liver cancer, the response rate of these therapies is still limited.

Consequently, there is still a demand for more treatment alternatives to improve the clinical results of patients with liver cancer. The discovery of additional molecular targets and the creation of other therapeutic strategies could lead to advancements in the treatment of liver cancer. The combination of molecular-targeted agents is likely to bring about a synergistic anticancer effect through targeting essential components within hepatocarcinoma cells. Recently, it was discovered that lysionotin could activate the Hippo pathway to overcome Yap-driven resistance to sorafenib in hepatocellular carcinoma [7], and also make liver cancer cells more sensitive to apoptosis by strengthening the ER stress-mediated pro-apoptotic pathway [8]. Moreover, in this study, RNAseq analysis was employed to statistically analyze the mRNA expression levels obtained from the whole transcriptome sequencing, and it was shown that differentially expressed genes were concentrated in the signaling pathways treated by lysionotin, such as the Hippo pathway, cell apoptosis, and autophagy.

Autophagy is a mechanism for cellular homeostasis, which promotes the proliferation and survival of advanced cancers through the degradation and recycling of organelles and proteins [9]. However, the detailed mechanisms and molecular events involved in this process are extremely complex. On the one hand, autophagy acts as a cytoprotective mechanism, offering protection against various diseases, especially tumors, cardiovascular disorders, neurodegenerative diseases, and infectious diseases. On the contrary, autophagy might also have a negative impact by exerting pro-survival effects on cancer cells or cell-killing effects on normal body cells [10].

In our study, we probed into the impacts of lysionotin on the growth, chemosensitivity, and apoptosis potential of HCC cells in vitro. Autophagy can be modulated by numerous signaling pathways, among which the AKT/mTOR pathways are of great significance in tumor initiation and progression. Our previous studies revealed that the activation of the AKT signaling has a crucial role in the growth and metastasis of HCC [11]. Moreover, whether lysionotin exerts its anticancer effects by regulating autophagy through the AKT/mTOR pathways.

2 Materials and methods

2.1 Reagents and antibodies

The biochemical reagent lysionotin (#3807) was purchased from Nature Standard (Shanghai, China). 3-Methyladenine (3-MA) (#HY-10219), Chloroquine (CQ) (#HY-17589A), Rapamycin (RAPA)(#HY-10219), MHY1485 (#HY-B0795) was purchased from MedChem Express (MCE). The following antibodies ATG12 (1:1000, #D88H11), Beclin-1 (1:1000, #D40C5), Anti-rabbit IgG (1:3000, #7074), Anti-mouse IgG (1:3000, #7076) were purchased from Cell Signaling Technology (Danvers, MA). P62 (1:5000, #18420-1-AP),mTOR (1:5000, #66888-1-Ig), phospho-mTOR (1:5000, #67778-1-Ig), phospho-AKT(Ser473) (1:5000, #66444-1-Ig), Lc3 (1:5000, #14600-1-AP) were purchased from proteintech (Wuhan,China); AKT (1:500, #bsm-33278 M) were purchased from Bioss(Beijing,China); β-actin (1:1000, #TA-09) was purchased from ZSGB-Bio(Beijing,China); Lipofectamine TM 3000 were purchased from Thermo Fisher Scientific. Hoechst 33258 (IH0060) was purchased from Solarbio Life Science.

2.2 Cell culture

Human hepatocellular carcinoma Hep3B and HepG2 cells were both cultivated in Dulbecco's Modifed Eagle's Medium (DMEM, Sigma D5796) supplemented with 10% fetal bovine serum (FBS, Excell FSP500) and 1% solution containing 10,000 IU/mL penicillin and 10,000 µg/mL streptomycin (Solarbio, T1320) and placed in an incubator at 37 °C with 5% CO_2. Observe cell status and adhesion and the cells with good growth status were selected for subsequent experiments.

2.3 Cell morphology

Select HepG2 and Hep3B cells in the logarithmic growth phase. Add trypsin for complete digestion, dilute to 3×10^4 cells/mL, and inoculate them in 12-well culture plates. After 24 h of continuous culturing, the cells were photographed and observed under an optical microscope in accordance with different experimental groups to observe the cell morphology and growth state.

2.4 Hoechst 33258 staining assay

Logarithmically proliferating HepG2 and Hep3B cells were sown in 12-well culture plates and cultivated for an additional 24 h. The cells were subjected to Hoechst 33258 staining solution and incubated at 37 °C for 5 min after 24 h treated by lysionotin. After that, the staining solution was removed, and the cells were rinsed three times with PBS. Then, the cell morphology, especially the proportion of bright blue nuclei, was inspected under a fluorescence microscope. All experiments were repeated three times.

2.5 Western blot analysis

HCC cells were collected and lysed on ice with RIPA lysis buffer. The protein concentration was measured by the BCA method. Subsequently, SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed for electrophoresis, and the proteins were transferred to PVDF membranes. Then, the PVDF membranes were blocked, and a series of processes like incubation with primary antibody, secondary antibody, and washing were conducted. Finally, the expression of related proteins was detected using ECL chemiluminescence solution.

2.6 Transmission electron microscopy assays (TEM)

For TEM analysis, cells were fixed with 2.5% glutaraldehyde at 4 °C for 2 h, followed by post-fixation with 1% osmium tetroxide in 0.1 M PB (pH 7.4) for 2 h at room temperature. Dehydration was performed using graded ethanol (50%, 70%, 80%, 90%, 100%) and 100% acetone, each for 15 min. Samples were then embedded, sectioned, and stained with 2% uranium acetate in alcohol (8 min, light-protected) and 2.6% lead citrate (8 min, CO_2-free), followed by overnight drying and embedding in Epon 816. Ultrathin sections were prepared using a Leica ultramicrotome and stained with uranium dioxyacetate and lead citrate. Imaging was conducted using a JEM-1400 TEM (JEOL, Japan).

2.7 mCherry-EGFP-LC3B system

Hep3B cells were transfected with mCherry-EGFP-LC3 to assess autophagy flux. After overnight incubation in confocal dishes, cells were treated under experimental conditions, fixed with absolute ethanol for 20 min, and washed with PBS three times. Imaging was performed using a LEICA Stellaris 5. Autophagosomes and autolysosomes were quantified based on yellow and red LC3 puncta in merged images, respectively.

2.8 RNAseq and data analysis

The Hep3B cells treated with or without 25 µM lysionotin for 24 h, and then the cells were gathered for RNA sequencing at BGI Genomics in China. Differentially expressed genes were identified using thresholds of fold change ≥ 2 and adjusted Q value < 0.05. Gene expression heatmaps were constructed and hierarchically clustered using the online bioinformatics platform (http://www.bioinformatics.com.cn/). Functional annotation analysis was performed through Gene Ontology (GO)

enrichment covering three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). Pathway enrichment analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Both analyses were implemented through the KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/kobas3). Gene Set Enrichment Analysis (GSEA) was subsequently performed to identify significantly enriched biological pathways using the GSEA 4.3 software.

2.9 PCR assay

The PCR procedure involves several critical steps. Initially, the DNA template, primers (Supplementary Table 1), Taq enzyme, dNTPs, and buffer are prepared and proportionally mixed to assemble the reaction system in PCR tubes, with triplicate replicates per tube. The basic process can be divided into three core steps of the cycle repetition, namely denaturation-annealing-extension. Each of the above three steps constitutes a cycle and is repeated 25–40 times. Post-amplification, products are analyzed using methods such as gel electrophoresis.

2.10 Statistical analysis

The results are presented in the form of mean ± SD. To ascertain the significant difference between the values in the two groups, one-way analysis of variance and post-hoc multiple comparisons Bonferroni tests were employed. Values with $P < 0.05$ were regarded as significant.

3 Results

3.1 Lysionotin regulates multiple molecular pathways that are involved in autophagy activation in HCC cells

The gene expression profiles of Hep3B cells were evaluated by RNAseq to understand the underlying molecular mechanisms through which lysionotin inhibited the proliferation of HCC cells. This technique analyzed 1305 differentially expressed genes. In total, 718 of these genes were down-regulated, whereas 587 genes were observed to be up-regulated (Fig. 1A).

The GO enrichment analysis method was used to explore the functions of different genes from three aspects: CC, MF, and BP. The top five enriched functions are displayed in Fig. 1B. The differentially expressed genes are mainly located in CC: non-membrane-bounded organelle, intracellular non-membrane-bounded organelle cytosol, membrane-enclosed lumen and cytoplasmic microtubule, MF: protein tyrosine/threonine phosphatase activity, kinase binding, protein kinase binding, enzyme activator activity, enzyme binding, and BP: integrated stress response signaling, hepatic biliary system development, liver development, semi-lunar valve development, intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (Fig. 1B).

In KEGG enrichment analysis, it was found that the MAPK signaling pathway, transcriptional mis-regulation in cancer, pathway in cancer and autophagy-related signaling pathways were significantly enriched in gene enrichment (Fig. 1C). Further analysis using the GO gene enrichment database revealed that GOCC results showed differential signals mainly enriched in autophagosome (Fig. 1E). GOBP analysis found that, apart from inhibiting cell growth, the pathways related to regulating autophagy were most significant. These included regulation of autophagy of mitochondrion, autophagy of mitochondrion and regulation of autophagosome maturation. Additionally, metabolism-related signaling pathways were also significant (Fig. 1D, F–H). GO analysis indicated that the mis-regulated genes showed enrichment for the gene set that was linked to the Autophagy (Fig. 1C) and many other genes that were enriched in "Autophagy", such as "ATG16L1, LAMP2, STX17, ATG2A, ATG16L2, C9orf72, ATG5, ATG9A, SNAP29, ATG13, ATG4D, NBR1, ATG12, LAMP1, NRBF2, ATG14, SQSTM1, AMBRA1, MAP1LC3B and ATG4B" (Fig. 1I). The PCR experiment confirmed these results (Fig. 1J). These findings suggest a potential association between lysionotin-induced autophagy signaling pathway activation and the observed inhibition of HCC cell growth.

3.2 Lysionotin induces autophagy in HCC cells

Next, we explored whether lysionotin could trigger autophagy in HCC cells. Firstly, Hep3B cells were treated with or without 25 μM Lys for 12 and 24 h. The outcomes of transmission electron microscopy revealed that the number of autophagosomes in the treated cells increased compared to the control group (Fig. 2A).

And then, logarithmic Hep3B cells (transfected with EGFP mCherry LC3 plasmid) were cultured for 10 days after lysionotin treatment, it was observed under laser confocal fluorescence microscopy that Lys significantly induced autophagic

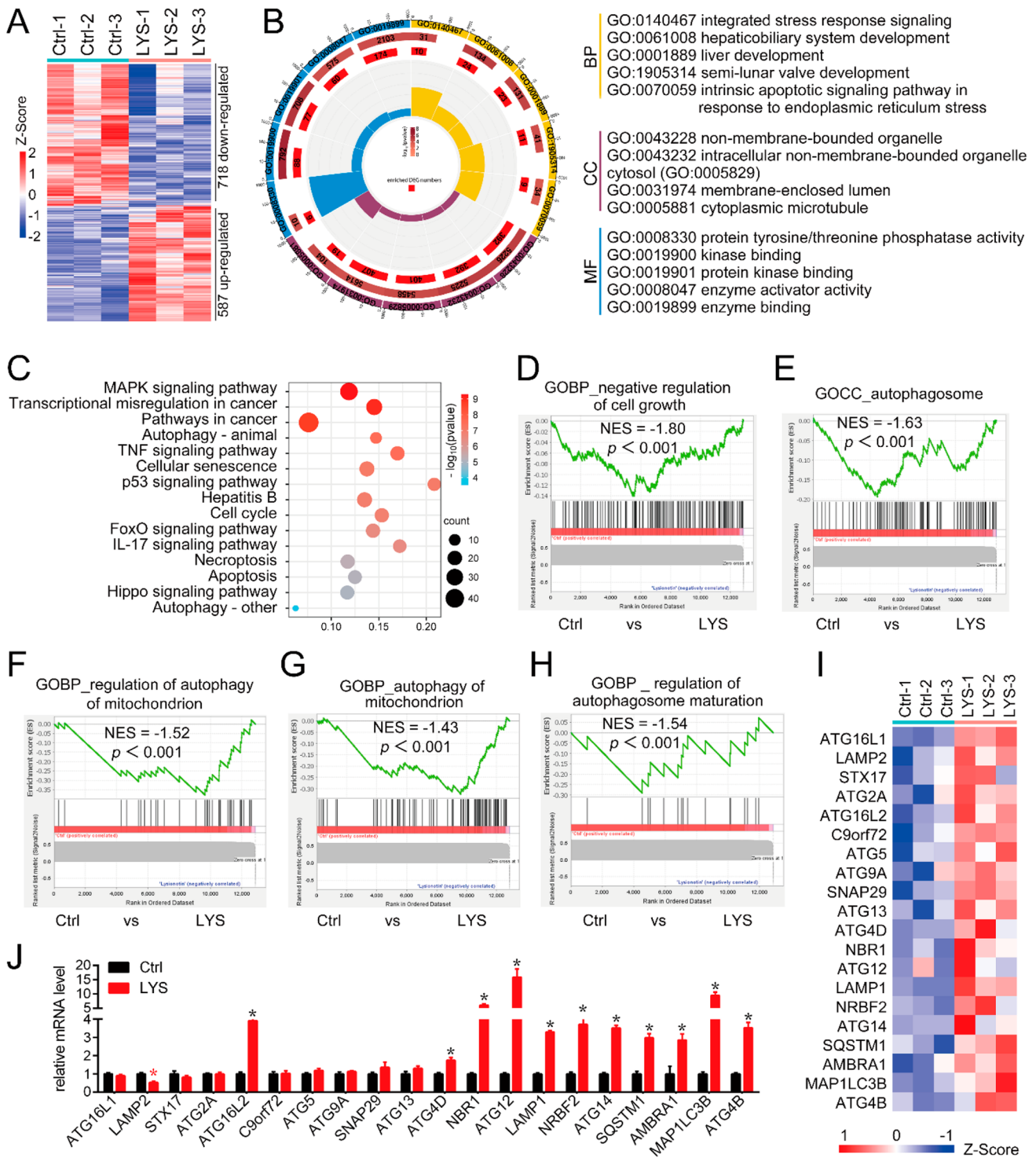


Fig. 1 LYS regulates multiple molecular pathways that are involved in autophagy in HCC cells. **A** The gene expression profiles were assessed using RNAseq in LYS-treated Hep3B cells. **B** The GO enrichment analysis method was used to explore the functions of different genes. **C** KEGG analysis showed enrichment for the gene set that was linked to the Autophagy. **D, E** Analysis the mis-regulated genes showed enrichment for the gene set that was linked to negative regulation of cell growth and autophagy. **F–H** GO-BP analysis the mis-regulated genes showed enrichment for the gene set that was linked to regulation of autophagy of mitochondrion, autophagy of mitochondrion and autophagosome maturation. **I–J** Statement these genes that were enriched in Autophagy and PCR experiment confirmed these genes

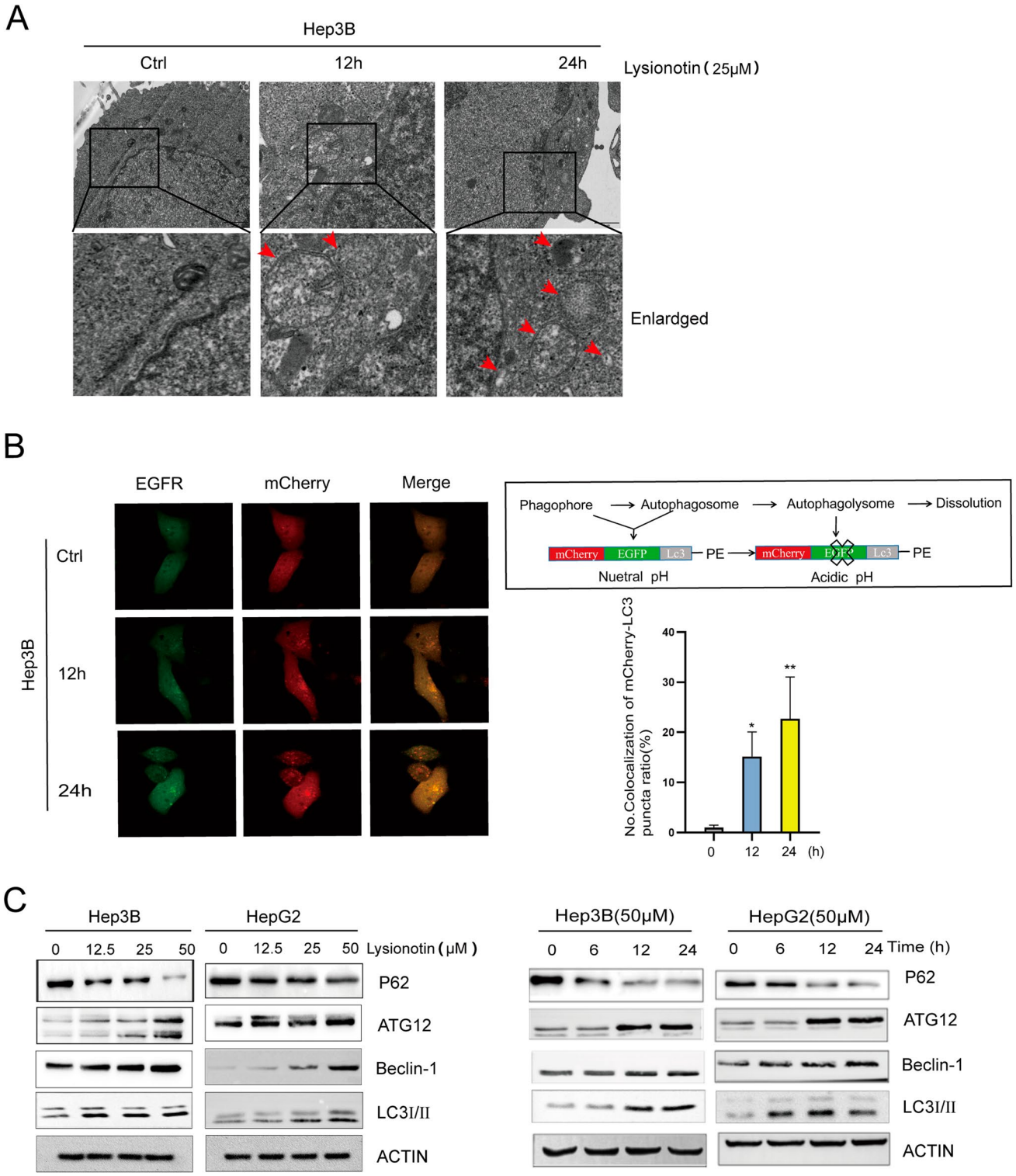


Fig. 2 LYS promoted the occurrence of autophagy in HCC cells. **A** Autophagy-specific structures were analyzed by transmission electron microscopy in LYS-treated cells. Arrows, autophagosomes. **B** Representative immunofluorescence images and quantification of the LC3B puncta in mCherry-EGFP-LC3B transfected Hep3B cells in the presence or absence of lys treatment for 12 h or 24 h. **C** Western blotting analysis of LC3-I, LC3-II, ATG12, Beclin-1 and P62 in HCC cells exposed to LYS; β-actin was regarded as internal reference

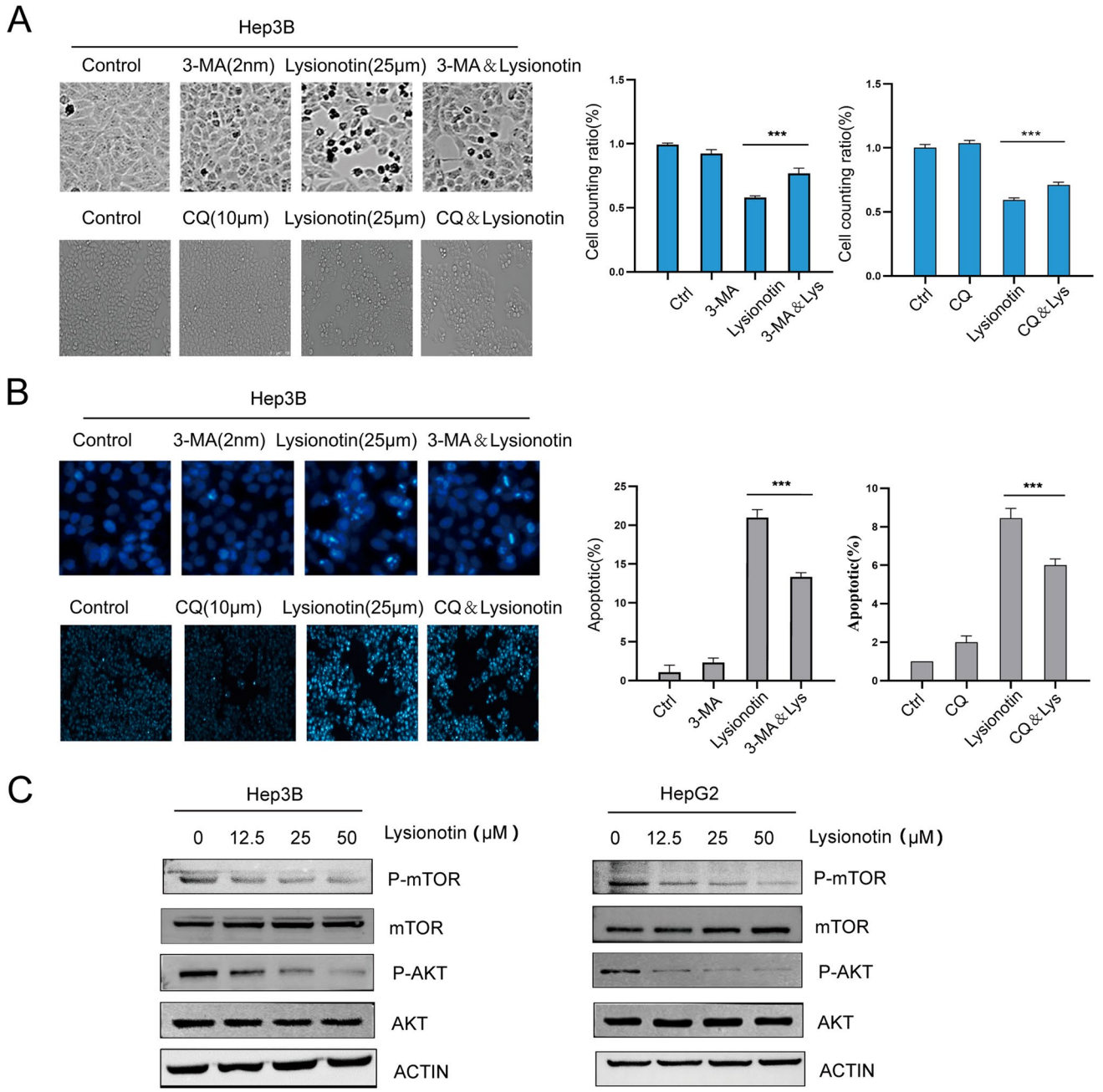


Fig. 3 LYS promotes HCC cell apoptosis via autophagy. **A** The cell survival rate of cells following the combination of LYS with or without 3-MA or CQ is determined by light microscopy ($\bar{x} \pm s$, $n = 3$). **B** The apoptosis rates of cells after the combination of LYS with or without 3-MA or CQ are measured by light microscope and Hoechst 33258 fluorescent staining ($\bar{x} \pm s$, $n = 3$) *P < 0.05, ***P < 0.001 compared with the control group. **C** Western blotting analysis of mTOR, P- mTOR, AKT, P-AKT in cells exposed to LYS; β-actin is taken as the internal reference

flow in Hep3 cells expressing the EGFP-mCherry-LC3 reporter gen (Fig. 2B). Lysionotin promotes the expression of autophagy pathway related proteins in liver cancer cells (Fig. 2C). In addition, using different concentrations (12.5, 25, 50 μM) After 24 h of Lys culture of Hep3B and HepG2 cells, compared with the control group, the expression levels of ATG12, Beclin-1 and Lc3-II proteins increased, while P62 decreased. The conversion of LC3- I (19 kD) to LC3- II (17 kD) and the downregulation of P62 are important markers of autophagy (Fig. 2C). Hep3B and HepG2 cells were cultivated with 50 μM lysionotin for diverse durations (0, 6, 12, 24 h), and western blot analysis demonstrated that, in contrast to the control group, the expression levels of ATG12 and Beclin-1 proteins in Hep3B and HepG2 cells increased, while P62 decreased,

Fig. 4 LYS activates autophagy and promotes HCC cell apoptosis through the AKT mTOR pathway. **A** Western blotting analysis of mTOR, P-mTOR, AKT, P-AKT and Lc3 in Hep3B cells exposed to LYS combinated with/without RAPA; β-actin was regarded as internal reference. **B** Western blotting analysis of mTOR, P-mTOR, AKT, P-AKT and Lc3in HepG2 cells exposed to LYS combinated with/without 1485. β-actin was regarded as internal reference. **C** the cell survival rate and apoptosis rate of Hep3B cells after the combination of LYS with or without RAPA are determined by optical microscope ($\bar{x} \pm s$, $n = 3$). **D** the cell survival rate and apoptosis rate of HepG2 cells after the combination of LYS with or without 1485 are measured by optical microscope ($\bar{x} \pm s$, $n = 3$)

suggesting the occurrence of autophagy (Fig. 2C). These results imply that lysionotin triggers autophagy processes in liver cancer cells, though contributions from lysosomal activity or alternative degradation pathways cannot be excluded.

3.3 Lysionotin promotes apoptosis by activating autophagy in liver cancer cells

In order to further verify that lysionotin promotes apoptosis of liver cancer cells by activating autophagy, we used a combination of autophagy inhibitors and Lys to inhibit autophagy in liver cancer cells from the formation phase of autophagosomes and the binding phase of autophagosomes and lysosomes, and observed the growth status of liver cancer cells. Autophagy inhibitor 3-MA can inhibit autophagy in liver cancer cells and reduce apoptosis. Hep3B cells were cultured separately and in combination with autophagy inhibitor 3-MA. Observe cell survival rate using light microscopy (Fig. 3A) and Hoechst 33258 staining (Fig. 3B). Compared with the control group, the Lys group had the most cell apoptosis, followed by the combination therapy group. The autophagy inhibitor CQ can also inhibit autophagy in liver cancer cells and reduce cell apoptosis. Hep3B cells were cultured separately and in combination with autophagy inhibitor CQ. The result is the same as above (Fig. 3A, B).

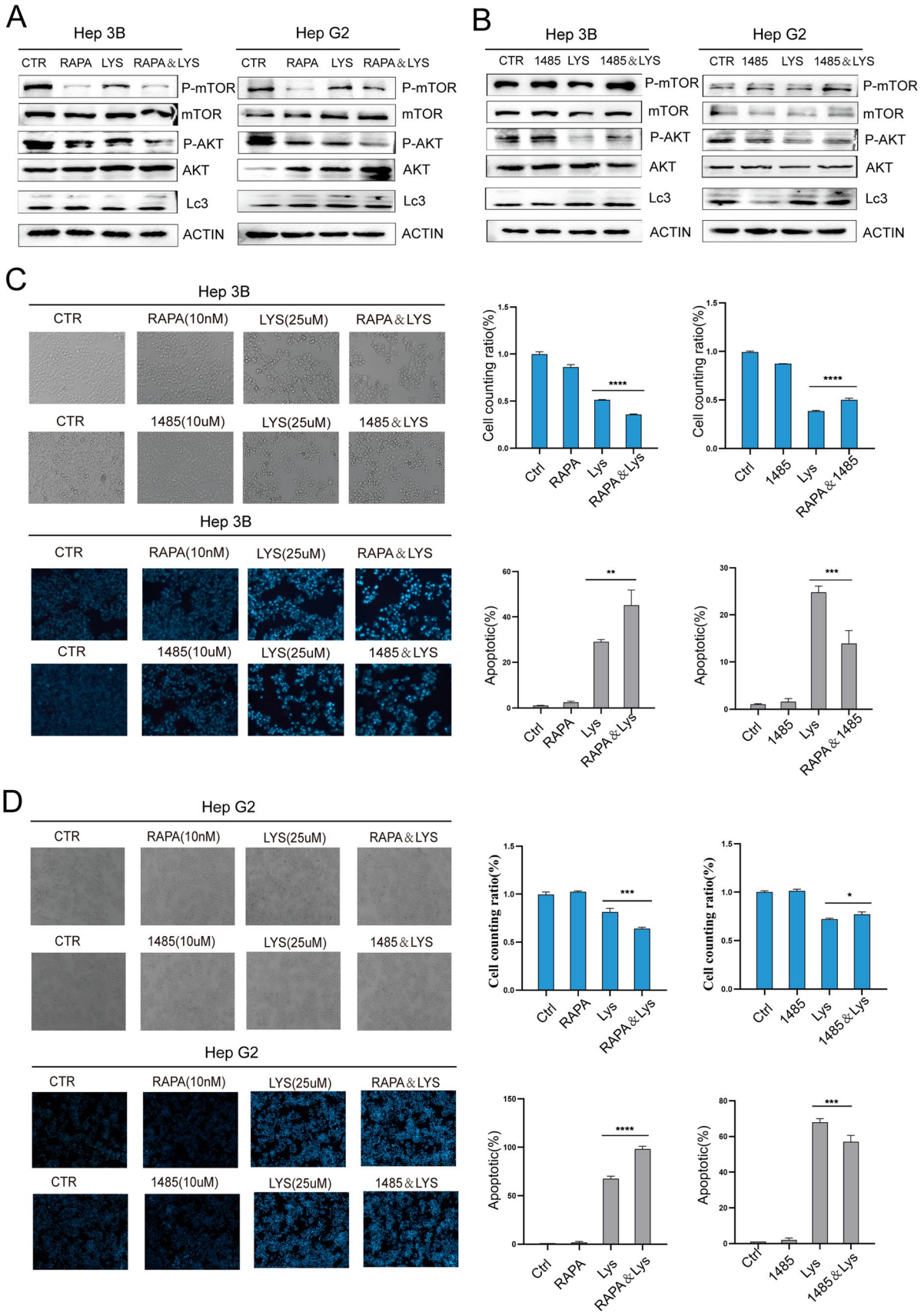
To investigate the molecular mechanisms of how lysionotin induces apoptosis and autophagy in HCC cells, western blot results found that lysionotin inhibited the activation of the mTOR-AKT pathway. Immunoblot analysis showed that compared with the control group, the expression levels of P-mTOR, and P-AKT proteins in Hep3B and HepG2 cells were reduced (Fig. 3C). This indicates that Lys can inhibit the activation of mTOR and AKT, autophagy related pathways. So, is the inhibition of the AKT-mTOR pathway by lysionotin related to lysionotin-induced autophagy?

3.4 Lysionotin activates autophagy by inhibiting the AKT mTOR pathway

Since lysionotin can inhibit the mTOR pathway and activate autophagy, used of mTOR inhibitors alone or in combination with lysionotin to find whether lysionotin activates autophagy in Hep3B and HepG2 cells by inhibiting the mTOR pathway. Immunoblot analysis showed that compared with the control group, the expression levels of P-mTOR and P-AKT proteins in the lysionotin group decreased, while the expression levels of Lc3 proteins increased. The Lys and RAPA combined group had the lowest expression levels of P-mTOR and P-AKT proteins, while the Lc3 protein expression level was the highest. We validated the use of mTOR activators and lysionotin alone or in combination, and found that lysionotin activates HCC cells autophagy by inhibiting the mTOR pathway (Fig. 4A). Immunoblot analysis showed that compared with the control group, the expression levels of p-mTOR and p-AKT proteins in the lysionotin group decreased, while the expression levels of Lc3 proteins increased. The expression levels of p-mTOR and p-AKT proteins in the Lys and 1485 combination groups were higher than those in the Lys group, while the expression levels of Lc3 proteins were lower than those in the lysionotin group (Fig. 4B). Based on this, it was further verified that lysionotin activates autophagy by inhibiting the mTOR pathway and induces HCC cells apoptosis. Observe cell survival rate using light microscopy (Fig. 4C, D) and Hoechst 33258 staining (Fig. 4C, D). The combined group of Lys and RAPA had the highest apoptosis rate; lysionotin group comes second, and lysionotin 1485 combined group comes second. In our results, we found that Lys could induce autophagic apoptosis in HCC cells by inhibiting the AKT -mTOR pathway.

4 Discussion

Autophagy is a tightly regulated process that plays a crucial role in maintaining cellular health and function. It involves the breakdown and recycling of damaged or unnecessary proteins and organelles, allowing cells to adapt to various stressors and maintain internal stability [12]. In addition to its role in preserving protein quality, autophagy also contributes to energy metabolism, immune response, and development. It serves as a protective mechanism by removing harmful substances from cells and promoting cell survival during adverse conditions. However, disruptions in autophagic processes or excessive autophagic activity can have detrimental effects on cells. Overactive autophagy may result in


A
Hep 3B
Hep G2
CTR RAPA LYS RAPA&LYS
P-mTOR
mTOR
P-AKT
AKT
Lc3
ACTIN
B
Hep 3B
Hep G2
CTR 1485 LYS 1485&LYS
C
Hep 3B
CTR RAPA(10nM) LYS(25uM) RAPA&LYS
CTR 1485(10uM) LYS(25uM) 1485&LYS
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the indiscriminate breakdown of essential cellular components, ultimately leading to cell death [13].Understanding the precise regulation of autophagy is essential for developing potential therapeutic approaches for diseases such as cancer, neurodegenerative disorders, and metabolic syndromes [14]. Research into modulating autophagy holds promise for improving human health by targeting this fundamental cellular process [15].

Autophagy has a complex impact on the development and advancement of tumors, serving as both a promoter of cell survival in certain situations and a trigger of autophagic cell death in specific cellular environments [16, 17]. As we known, hypoxia has been demonstrated to induce autophagy in hepatocellular carcinoma (HCC) cells [18]; however, the impact of hypoxia-induced autophagy on HCC progression exhibits significant variability depending on the context. For instance, epidemiological studies indicate that the incidence of HCC among individuals residing in high-altitude, hypoxic environments is markedly lower compared to those living in low-altitude plains. Conversely, the survival rate of HCC patients in hypoxic regions is significantly higher than that in plain areas [19],On the other hand, extensive preclinical research has revealed that moderate hypoxia can influence various aspects of HCC biology, including cellular aging, apoptosis, metabolic reprogramming, tumor microenvironment remodeling, and immune modulation, ultimately promoting tumor proliferation, invasion, and metastasis [20].

In the present study, we found that RNAseq analysis that autophagy activated in HCC cells after treatment by Lysionotin. TEM and laser confocal fluorescence microscopy results showed an increase in autophagosomes in the treated cells compared to the control group indicating that Lys significantly induced autophagic flow. Western blots showed that the level of LC3-II obviously increased after treated by Lys, ATG12 also up-regulated, these proteins as marker proteins of autophagy, indicating that Lys may activate autophagy. It is interesting that P62 was down-regulated and Beclin-1 up-regulated. Although P62 was the first autophagy adaptor to be identified, it stands out from other adaptors (NBR1, TAX1BP1, NDP52, and OPTN) in that, with the exception of NBR1, it also serves as a central hub by interacting with key signaling proteins through specific structural elements [21]. Autophagy plays a crucial role in regulating P62 levels as it is continuously degraded through non-selective autophagy via its LIR domain binding to LC3 on autophagosome membranes [21], in addition to its involvement in selective autophagy, P62 acts as an important pro-oncogenic regulator due to its function as a signaling hub. Notably, high levels of P62 protein in epithelial cells are both necessary and sufficient for inducing oncogenic transformation independent of its functions related to autophagy [22]. This may be another factor that Lys inhibits P62 independent of autophagy. These indicates that a key role of autophagy in suppressing tumors is to prevent P62-mediated tumor initiation and malignant transformation. This has important implications for the potential development of therapeutic strategies for cancer by targeting autophagy or P62-regulated signaling pathways.

Beclin1, a critical protein involved in the autophagic process, interacts with various binding partners and regulatory proteins related to both autophagy and apoptotic cell death pathways. It plays an important role in controlling the communication between these two types of cell death and is necessary for regulating a wide range of physiological and pathophysiological conditions [23]. In this study, we found Lys promoted the expression of Beclin1, this may be one of the reason of Lys induced HCC cells apoptosis related to autophagy activation. More, when HCC cells were pretreated with 3-MA or CQ, inhibitors of autophagy, the effects of Lys on apoptosis and cell viability ability were markedly attenuated, indicating that Lys acts as an activator of autophagy to induce apoptosis in HCC cells.

Recent data has shown that P62, located on lysosomes and capable of binding Raptor and the Rag proteins, regulates mTORC1 activity. Interestingly, deficiency of P62 in various cell systems impairs the recruitment of mTORC1 to the lysosomes and its activation in response to amino acids [24] and Tsc1 ablation [22]. As we known, the AKT/mTOR pathway is closely related to the activation of autophagy in various tumors [25–28]. In this study, we discovered that lysionotin inhibits the activation of the AKT/MTOR signaling pathway, which may be a primary mechanism underlying lysionotin-induced autophagy. Furthermore, when lysionotin was combined with the mTOR inhibitor RAPA or activator 1485, a significant increase in apoptosis was observed after combination with RAPA. Conversely, apoptosis was reversed following mTOR activation in combination with 1485. This suggests a novel approach for the combined use of certain autophagy inhibitors in cancer therapy. Collectively, we found that Lys is a potential natural anti-cancer product which has an inhibitory effect on HCC tumorigenesis and makes HCC cells more sensitive to RAPA treatment. These discoveries provide solid evidence about the potential of Lys as therapeutic alternatives for the treatment of HCC.

While this study provides evidence for the antitumor potential of Lys in HCC, several limitations warrant cautious interpretation of the findings. Firstly, although our results revealed modulation of PI3K/AKT/mTOR signaling pathways, the precise molecular targets mediating Lys's therapeutic effects remain elusive. Our current data cannot distinguish between direct target engagement and secondary regulatory effects, particularly regarding potential interactions with VEGFR [29], PD-1/PD-L1 [30], c-MET [31], Wnt/β-catenin [32] and effects on tumor-associated lymphatic vessel density (LVD) [33]. Secondly, the effects of Lys on the xenografted tumor model in nude mice or patient-derivedxenografts (PDX)

model needs to be further verified. Future work should prioritize: (1) Target deconvolution using photoaffinity labeling coupled with CRISPR/Cas9 functional screening; (2) Longitudinal assessment in immunocompetent HCC models with monitoring of metastatic potential; (3) Quantitative systems pharmacology modeling to integrate multi-omics datasets (proteome/ phosphoproteome/ transcriptome). Addressing these gaps will elucidate the therapeutic window of Lys and its potential synergies with existing targeted therapies, ultimately guiding rational clinical translation. These above aspects will provide more directions for thinking regarding the study of the antitumor effect of the Lys.

5 Conclusions

Our in vitro findings suggest that lysionotin warrants further investigation as a potential therapeutic agent for HCC, particularly in combination with mTOR inhibitors like RAPA. However, preclinical validation in animal models and identification of direct molecular targets are essential next steps. Comprehending the function of autophagy in diverse stages of tumor formation holds significant importance when the domain is striving to determine whether to develop autophagy inhibitors or inducers as pharmacological substances to target autophagy in cancer. Furthermore, the influence of autophagy on the therapeutic effect of epigenetic drugs or drugs targeting post-translational modification has also been deliberated, offering perspectives on the combination with autophagy activators or inhibitors in the treatment of clinical disorders.

Author contributions MJ. L., YC. Y., XX. W., WW. Z and Q. W. were involved in the experimental design, conducted the experiments, analyzed the data, and composed the manuscript. CY. Y., X.L., P. S and BH L performed the experiments and analyzed the data.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

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