

Metformin Induces Autophagy via the AMPK-mTOR Signaling Pathway in Human Hepatocellular Carcinoma Cells

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Background: Metformin may exert the anticancer effect on multiple types of cancers and some potential mechanisms have been suggested. Our study was designed to determine the effect of metformin on the cell autophagy and autophagic flux via the AMPK-mTOR signaling pathway in human hepatocellular carcinoma (HCC) cells.

Methods: MHCC97H and HepG2 cell lines were cultured and treated without and with metformin at various concentrations (2, 5, 10 and 20 mM) for 48 h. Then, 10 mM was determined as the optimal concentration and the HCC cells were treated with metformin for 12, 24, 48, and 72 h. MTT assay was used to assess the cell viability and Western blotting was used to determine the expression of proteins (LC3-II, p62, phospho-AMPK α , phospho-mTOR, mTOR, phospho-p70 S6 Kinase, p70 S6 Kinase, PARP1, Caspase-9 and Caspase-3). Autophagy inhibitor 3-methyladenine, EGFP-LC3 and mCherry-GFP-LC3B plasmid transfection were used for further study.

Results: Metformin inhibited significantly the viability of MHCC97H and HepG2 cells in a dose- and time-dependent manner. For the apoptotic properties, activation of Caspase-9 and Caspase-3 and PARP cleavage were not observed after treatment with metformin. MHCC97H cells were transfected with a EGFP-LC3 plasmid and treatment with metformin could lead to the increased level of LC3-II and decreased level of p62. In metformin-induced autophagy, AMPK expression was activated, and the phosphorylation levels of mTOR and p70 S6 Kinase were inhibited. Metformin treatment and mCherry-GFP-LC3B plasmid transfection showed that metformin could induce the autophagic flux. 3-Methyladenine (3-MA) partly abolished this effect.

Conclusion: Metformin could induce the autophagy, autophagic flux, and activate the AMPK-mTOR signaling pathway in human HCC cells.

Keywords: metformin, hepatocellular carcinoma, autophagy, autophagic flux, AMPK-mTOR signaling pathway

Introduction

Hepatocellular carcinoma (HCC), one of the common malignancies of digestive system, is the third most common cause of cancer death worldwide.^{1,2} In China, the mounting annual incidence is particularly high and the number is estimated as 40 per 100 000 persons per year.³ Some definite risk factors for HCC have been identified, including HBV infection, HCV infection, liver cirrhosis, heavy alcohol consumption, and aflatoxin exposure.^{2,4} In recent years, there are abundant evidences showing that diabetes mellitus (DM) is a confirmed risk factor for HCC and

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diabetic patients have a higher incidence of HCC.^{5,6} According to the 8th IDF Atlas, 425 million people have suffered from diabetes in 2007 and the number will rise to 629 million by 2045.⁷

Metformin (1,1-dimethylbiguanide hydrochloride), a biguanide derivative, is one of the most used first-line anti-hyperglycemic drugs for type 2 DM.³ Epidemiological studies have demonstrated that treatment with metformin can reduce the risk of some cancers, such as HCC, breast cancer, colorectal cancer and pancreatic cancer.^{8–10} For the association of metformin use with HCC risk, we have published one meta-analysis which included seven studies and 16,549 diabetic patients.³ Our overall analysis showed that diabetic patients with metformin use had a significantly reduced risk of HCC (relative risk 0.24, 95% confidence interval 0.13–0.46). These results were supported by experimental studies and multiple potential anti-cancer mechanisms of metformin were proposed,^{3,8–10} including inhibition of cell proliferation and hepatic gluconeogenesis, activation of AMPK, and modulation of microRNAs expression.

Autophagy, a mechanism by which the cells try to survive, plays important biological roles in the initiation and progression of tumors.¹¹ The AMP-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling pathway is well known to be associated with autophagy and AMPK can promote the initiation of autophagy.¹² Activation of AMPK can result in the inhibition of mTOR, which is commonly activated in malignant cells.^{3,12} Some studies suggested that metformin might exert the anticancer effect by regulation of the AMPK-mTOR signaling pathway.^{13,14} Our study was designed to determine the effect of metformin on the cell autophagy and autophagic flux via the AMPK-mTOR signaling pathway in human HCC cells.

Materials and Methods

Reagents and Antibodies

Metformin, PMSF, leupeptin and aprotinin were purchased from Sigma (St. Louis, MO, USA). Acrylamide, methylene bisacrylamide, sodium dodecyl sulfonate (SDS), Tris base, ammonium persulfate and Tween-20 were obtained from Amresco (Solon, OH, USA). Fetal bovine serum and DMEM were purchased from HyClone (Logan, UT, USA). Antibodies against p62, phospho-AMPK α (Thr172), phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 Kinase (Thr421/Ser424), p70 S6 Kinase and β -actin were obtained

from Cell Signaling (Danvers, MA, USA). Antibodies against PARP1, Caspase-9 and Caspase-3 were purchased from Abcam (Cambridge, MA, USA). Antibody against microtubule-associated protein 1 light chain 3 II (LC3-II) was from GeneTex (Irvine, CA, USA). Anti-rabbit/mouse goat immunoglobulin G-horseradish peroxidase (HRP) secondary antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). PVDF membrane was purchased from Millipore (Billerica, MA, USA). ECL Plus immunoblotting detection kit was from Bio-Rad (Hercules, CA, USA). Other routine reagents were obtained from Zhongshan (Beijing, China).

Human HCC Cells and Culture

Our research project was approved by the Clinical Research Ethics Committee of China–Japan Friendship hospital. High metastatic human HCC MHCC97H cell lines were purchased from Beijing BiYao Biotech Co., Ltd. (Beijing, China). The human HCC HepG2 cell lines, which had been authenticated by STR profile, were obtained from the Biochemistry Department of the Health Science Center of Peking University and approved by the Clinical Research Ethics Committee of China–Japan Friendship hospital. MHCC97H and HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/L glucose) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin sulfate, and maintained at 37°C in a humidified 5% CO₂ atmosphere.

MTT Assay

MTT assay was used to assess the cell viability and it was performed according to the manufacturer's instructions.¹⁵ Briefly, MHCC97H and HepG2 cells in the logarithmic growth phase were isolated and digested with 0.25% trypsin cell digestive fluid. After a single cell suspension was prepared, the cells were seeded onto 96-well flat-bottom microtiter plates at a density of 6×10^3 cells per well. HCC cells in the plates were pre-incubated overnight, allowed to adhere, and treated with metformin at various concentrations (0, 2, 5, 10 and 20 mM) for 48 h and 10 mM was selected as the optimal concentration. Then, they were treated with metformin (10 mM) for 24, 48, and 72 h. Six wells for each group, zero-adjustment well and control group were also set. After the treatments, 10 μ L of MTT reagent (5 mg/mL) was added to each well and the incubation was continued for 4 h. For each well, a total of 150 μ L DMSO was added and the absorbance value was

measured spectrophotometrically at a 570 nm wavelength. All the experiments were performed in triplicate and repeated thrice.

Western Blotting Analysis

Western blot analysis was used to determine the expression of proteins (LC3-II, p62, phospho-AMPK α , phospho-mTOR, mTOR, phospho-p70 S6 Kinase, p70 S6 Kinase, PARP1, Caspase-9 and Caspase-3). The experiments were repeated thrice. MHCC97H and HepG2 cells were lysed by adding modified RIPA buffer [50 mM Tris-HCl (pH7.5), 1% Triton-100, 150 mM NaCl, 2 mM EDTA, 1 mM sodium vanadate, 50 mM NaF, 1 mM PMSF, 1 mg/mL aprotinin, 1 mM leupeptin]. The supernatant was collected and bicinchoninic acid protein assay was used to determine the concentration of protein samples. Equal amounts (40 μ g) of protein samples were loaded onto SDS-containing 12% polyacrylamide gel and transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were probed with different primary antibodies, including LC3-II (1:1000), p62 (1:1000), PARP1 (1:1000), Caspase-9 (1:1000), Caspase-3 (1:1000), phospho-AMPK α (Thr172) (1:1000), phospho-mTOR (Ser2448) (1:1000), mTOR (1:1000), phospho-p70 S6 Kinase (Thr421/Ser424) (1:1000) and p70 S6 Kinase (1:1000). Secondary antibodies were used at a dilution of 1:5000 (anti-mouse) or 1:2000 (anti-rabbit). Antibody against LC3-II was obtained from GeneTex (Irvine, CA, USA). Antibodies against PARP1, Caspase-9 and Caspase-3 were purchased from Abcam (Cambridge, MA, USA). Antibodies against p62, p-AMPK α , p-mTOR, mTOR, p-p70 S6Kinase and p70 S6 Kinase were obtained from Cell Signaling (Danvers, MA, USA).

Plasmids Transfection

To further investigate the effect of metformin on autophagy in HCC, MHCC97H cells at approximately 60–70% confluence were transfected with a EGFP-LC3 expression plasmid using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).¹⁶ After incubation for 24 h, they were treated with metformin and observed. Moreover, MHCC97H cells were transfected with a mCherry-GFP-LC3B expression plasmid to investigate the effect of metformin on the formation of autophagosome and autolysosome, which can reflect the status of autophagic flux in HCC cells.

Confocal Laser Scanning Microscope

Before observation, HCC cells were fixed with 4% formaldehyde for 15 min, and cell nuclei were counterstained with DAPI. An Olympus FV500 confocal laser scanning microscope was used to obtain the images. Five non-overlapping fields were observed and percentages of cells with punctate EGFP-LC3 structures (green) were calculated. For the results of transfection with mCherry-GFP-LC3B, yellow fluorescence signals indicate the change of autophagosome and red fluorescence signals represent the change of autolysosome. Both yellow and red signals are increased simultaneously, indicating that autophagic flux is induced, whereas only yellow signals are increased, indicating that autophagic flux is inhibited. All the experiments were repeated thrice.

Results

Metformin Inhibits Cell Viability and Induces Autophagy in HCC Cells

Autophagy was associated with cell viability and death. Before we investigated the effect of metformin on cell autophagy, MTT assay was used to assess the cell viability by metformin in HCC MHCC97H and HepG2 cells (Figure 1A and B). MHCC97H and HepG2 cells were treated with metformin at various concentrations (2, 5, 10 and 20 mM) for 48 h. As Figure 1A showed, we found that the HCC cell viability could be inhibited significantly. The inhibitory rate was about 50% when the cells were treated with 10 mM metformin. Then, 10 mM was determined as the optimal concentration and the cells were treated with metformin for 24, 48 and 72 h (Figure 1B). The results showed that metformin inhibited the HCC cell viability in a time-dependent manner.

Autophagy and apoptosis are two basic physiologic processes to maintain the cellular homeostasis. PARP1, Caspase-9 and Caspase-3 were detected by Western blot analysis to determine the apoptotic properties of metformin (Figure 1C and D). MHCC97H and HepG2 cells were treated with metformin (10 mM) for 12, 24, 48, and 72 h. The positive control is treated with staurosporine. We found that activation of Caspase-9 and Caspase-3 and PARP cleavage was not observed after treatment with metformin.

To determine the effect of metformin on cell autophagy, autophagy-associated protein expression of LC3-II and p62 was detected by Western blot analysis in MHCC97H and HepG2 HCC cells (Figure 2C and E).

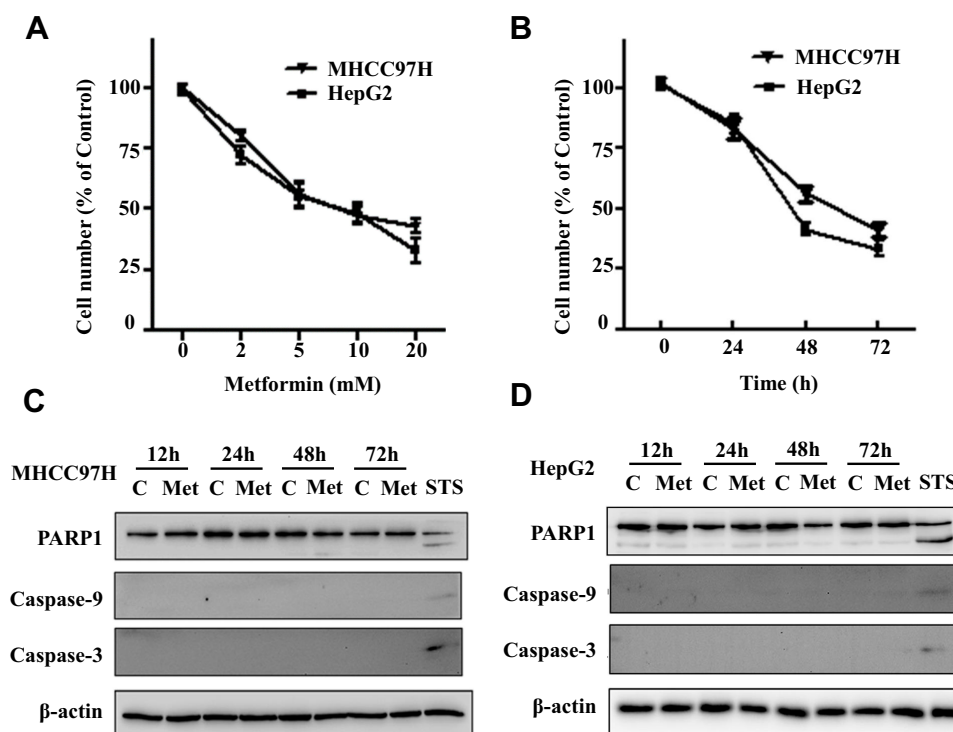


Figure 1 Metformin inhibits cell viability and the measurement of apoptotic properties of metformin in HCC cells. **(A)** MHCC97H and HepG2 cells were treated with metformin at various concentrations (2, 5, 10 and 20 mM) for 48 h and 10 mM was selected as the optimal concentration. MTT assay was used to assess the cell viability. **(B)** MHCC97H and HepG2 cells were treated with metformin (10 mM) for 24, 48, and 72 h. Data were expressed as means \pm standard deviation. **(C and D)** The apoptotic properties of metformin were measured in MHCC97H and HepG2 HCC cells. PARP1, Caspase-9 and Caspase-3 was detected by Western blot analysis. The "C" indicates the blank control group without treatment, the "Met" indicates the groups treated with metformin, and the "STS" indicates the positive control group which is treated with staurosporine. All the experiments were repeated thrice.

Abbreviations: C, control; h, hour; Met, metformin; STS, staurosporine.

The cells were treated with metformin (10 mM) for 12, 24, 48, and 72 h. P62 is a substrate of autophagy and it can be degraded by autolysosomes.¹⁷ Our results found that the p62 protein expression was decreased significantly, indicating that metformin could induce the autophagy in MHCC97H and HepG2 cells. Protein LC3-II is involved in the formation of autophagosomes and serves as one of autophagic markers.¹⁷ For the expression of LC3-II, we observed that it was increased in MHCC97H cells whereas it was decreased in HepG2 cells (Figure 2C and E). We deduced that metformin might have no obvious effect on the formation of autophagosomes in HepG2 cells, but it could affect any other pathway, such as autophagy flux, which could lead to the decreased LC3-II expression.

EGFP-LC3 Plasmid Transfection and Metformin-Induced Autophagy

MHCC97H cells were transfected with a EGFP-LC3 plasmid to further investigate the effect of metformin on the formation of autophagosomes (Figure 2A–D). Cells were treated with metformin (5, 10 mM) for 48 h and the results

showed that percentages of cells with punctate EGFP-LC3 structures (green fluorescent signals) were increased significantly (Figure 2A and B). Expression of LC3-II and p62 in MHCC97H cells was detected by Western blotting. As shown in Figure 2C and D, treatment with metformin could lead to the increased level of LC3-II expression and decreased level of protein p62 in a dose- and time-dependent manner. These results indicated that metformin could induce the formation of autophagosomes and the occurrence of autophagy.

Activation of AMPK in Metformin-Induced Autophagy

AMPK-mTOR signaling pathway is well known to be associated with cell autophagy and AMPK can promote the initiation of autophagy.¹² MHCC97H cells were treated with metformin (5, 10 and 20 mM) for 24 and 48 h (Figure 3A). The phosphorylation level of AMPK (Thr172) was analyzed by Western blot. We observed that, compared with the control, expression level of p-AMPK α (Thr172) was increased

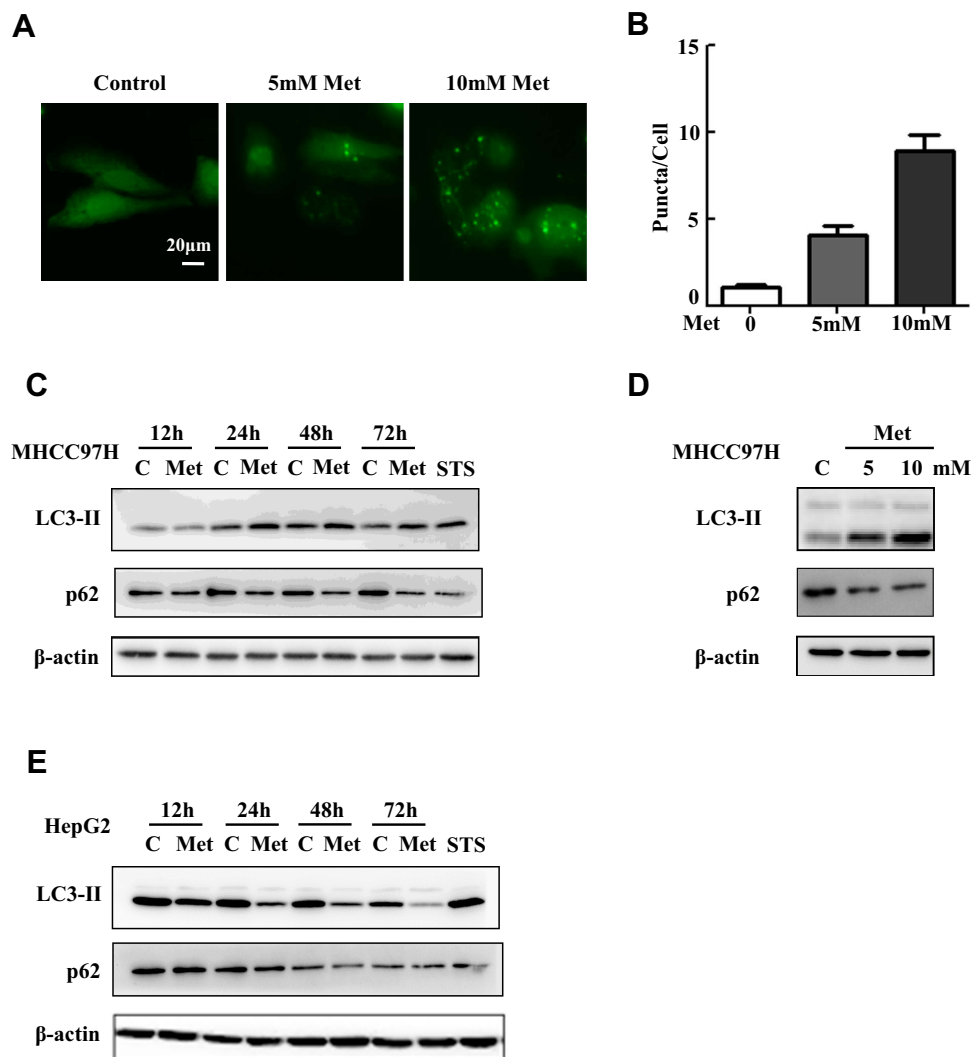


Figure 2 EGFP-LC3 plasmid transfection and metformin-induced autophagy in MHCC97H cells. (A and B) MHCC97H cells were transfected with a EGFP-LC3 plasmid. After incubation for 24 h, they were treated without or with metformin (5 and 10 mM) for 48 h and were visualized with the confocal laser scanning microscope (original magnification $\times 63$, scale bar, 20 μ m). The representative column diagrams showed the results and data were expressed as means \pm standard deviation. (C and D) MHCC97H cells were treated with metformin (10 mM) for 12, 24, 48, and 72 h (C), and treated with metformin at various concentrations (5 and 10 mM) for 48 h (D). Expression of LC3-II and p62 in MHCC97H cells was detected by Western blot analysis. (E) HepG2 HCC cells were treated with metformin (10 mM) for 12, 24, 48, and 72 h, and LC3-II and p62 was detected by Western blot analysis. The "C" indicates the control group, the "Met" indicates the metformin-treated groups, and the "STS" indicates the group which is treated with staurosporine. All the experiments were repeated thrice.

Abbreviations: C, control; h, hour; Met, metformin; STS, staurosporine.

significantly after treatment with metformin, indicating that AMPK expression was activated.

Metformin Reduced the Phosphorylation Levels of mTOR and P70 S6 Kinase

mTOR regulates cell growth and autophagy, and cell autophagy is inhibited by mTOR expression.¹² MHCC97H cells were treated with metformin at the above-mentioned concentrations for 72 h and the expression levels of phospho-AMPK α (Thr172), phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 Kinase (Thr421/Ser424) and p70 S6 Kinase were measured by Western blot (Figure 3B). The

results showed that, with the enhancement of AMPK activity, treatment with metformin could significantly reduce the phosphorylation levels of mTOR and p70 S6 Kinase expression.

Autophagy Inhibitor 3-MA Partly Abolished Metformin-Induced Autophagic Flux

Ubiquitin-associated protein p62 is usually used to monitor the autophagic flux.¹⁸ An increased level of p62 expression indicates that the autophagic flux is inhibited, whereas a decreased level indicates that the autophagic flux is

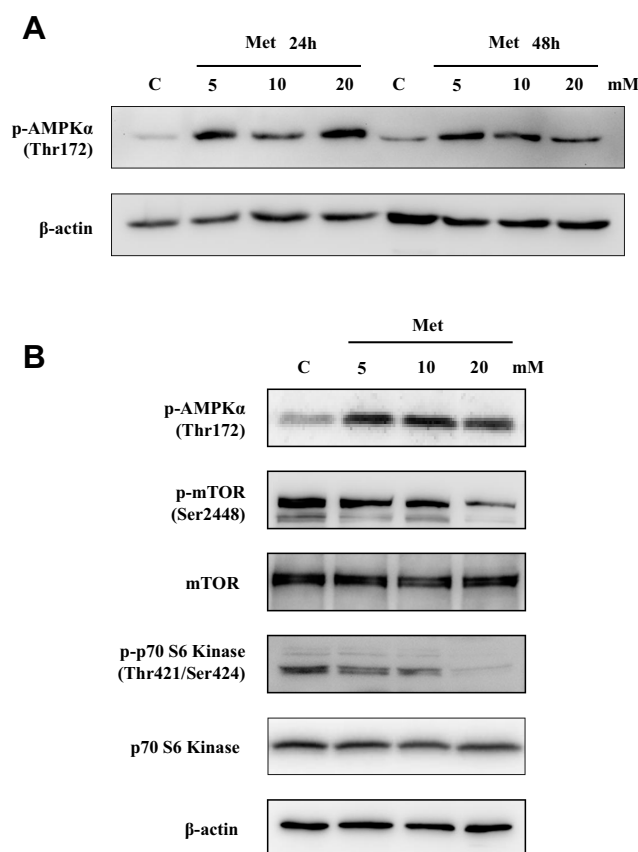


Figure 3 Metformin induces autophagy via the AMPK-mTOR signaling pathway in MHCC97H cells. **(A)** MHCC97H cells were treated with metformin (5, 10 and 20 mM) for 24 and 48 h. Cell lysates were separated by SDS-PAGE and the phosphorylation level of AMPK was analyzed by Western blot. **(B)** MHCC97H cells were treated with metformin at the above-mentioned concentrations for 72 h and the expression levels of phospho-AMPKα (Thr172), phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 Kinase (Thr421/Ser424) and p70 S6 Kinase were measured by Western blot. The "C" indicates the control group without treatment, and the "Met" indicates the groups treated with metformin. All the experiments were repeated thrice.

Abbreviations: C, control; h, hour; Met, metformin.

induced. As shown in Figure 2C–E, metformin could lead to decreased level of protein p62 in a dose- and time-dependent manner, indicating that metformin could induce the autophagic flux in MHCC97H and HepG2 cells. To further investigate metformin and autophagic flux, autophagy inhibitor 3-methyladenine (3-MA) was used and MHCC97H cells were pretreated with 3-MA (10 mM) for 2 h (Figure 4A). Compared with the expression level of p62 simply treated with metformin, the combination of 3-MA and metformin could lead to the increased level of p62 and decreased level of LC3-II expression.

mCherry-GFP-LC3B Plasmid Transfection and Metformin-Induced Autophagy

MHCC97H cells were transfected with a mCherry-GFP-LC3B plasmid to further study the effect of metformin on

the autophagic flux (Figure 4B). Two fluorescent signals could be observed with the confocal microscopy. Yellow signals indicate the change of autophagosome and red signals represent the change of autolysosome. Increasing level of both yellow and red signals indicates that autophagic flux is induced. We found that treatment with metformin could induce the autophagic flux.

Discussion

Metformin is one widely used first-line anti-hyperglycemic drug and abundant evidences have demonstrated that metformin treatment can reduce the risk of some cancers. Considering the association of autophagy with the initiation and progression of cancers, our study was designed to determine the effect of metformin on the autophagy and autophagic flux via the AMPK-mTOR signaling pathway in human HCC cells. We found that metformin could induce the autophagy and autophagic flux, lead to the increased level of LC3-II and decreased level of p62, activate the AMPK expression, and inhibit the phosphorylation levels of mTOR and p70 S6 Kinase.

Our result that metformin could induce the autophagy of HCC cells was consistent with some previous published researches.^{19,20} Tsai et al found that metformin could activate autophagy in Huh7 cells and the underlying mechanisms in this process were suggested.¹⁹ They showed that metformin treatment led to a reduced degradation of Src-mediated CCAAT/enhancer-binding protein delta (CEBPD) protein and an increased level of CEBPD-regulated LC3-II and ATG3 gene transcription. Another study was designed to determine the effect of combination of sorafenib and metformin on the autophagy via the mTOR pathway in HCC cells.²⁰ The authors discovered that metformin could induce the activation of mTORC2 and suppress the mTORC1 and MAPK pathway, which may be responsible for the induction of autophagy.

However, there are still dissenting voices on the association of metformin with cell autophagy, for example, this following study which was designed to investigate the effect of metformin on autophagy and apoptosis in H4IIE rat HCC cells.¹⁵ The author found that metformin treatment could reduce the viability of H4IIE cells, stimulate the pro-apoptotic events (nuclear condensation, hydrolysis of intact poly ADP ribose polymerase and caspase-3), but decrease the expression levels of autophagy-related proteins (LC3B, beclin-1, Atg3, Atg5, Atg7, and Atg12).¹⁵ As a researcher, the author naturally knew that metformin had been reported to induce autophagy in various types of

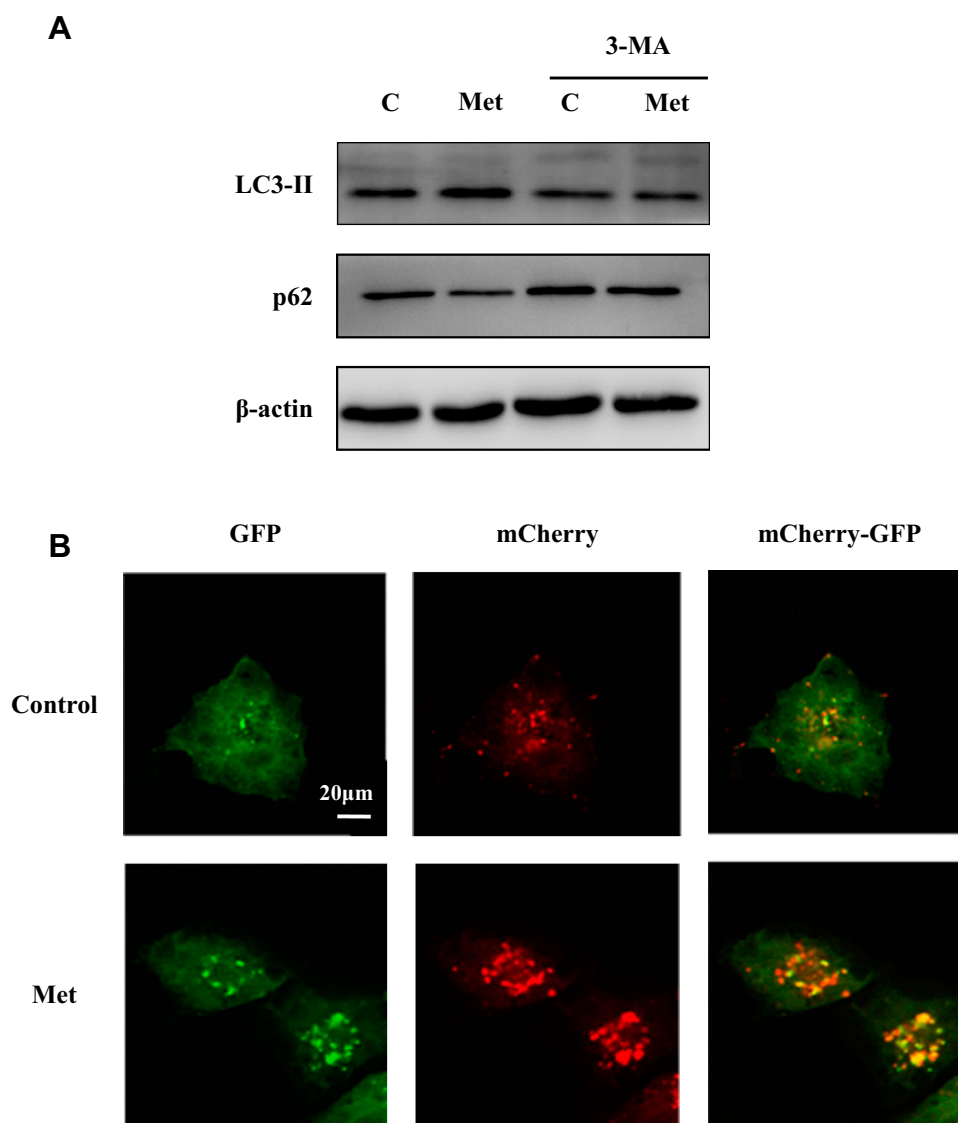


Figure 4 Autophagy inhibitor 3-methyladenine (3-MA) and mCherry-GFP- LC3B plasmid transfection in metformin-induced autophagic flux. **(A)** MHCC97H cells were pretreated with 3-MA (10 mM) for 2 h, followed by treatment with metformin (10 mM) for 48 h. The LC3-II turnovers and p62 level were detected by Western blot. **(B)** MHCC97H cells were transfected with a mCherry-GFP-LC3B plasmid, followed by treatment with metformin (10 mM) for 48 h. Representative fluorescent images were visualized with confocal microscopy (original magnification $\times 63$, scale bar, 20 μm). Yellow signals indicate the change of autophagosome and red signals represent the change of autolysosome. Increasing level of both yellow and red signals simultaneously indicates that autophagic flux is induced in MHCC97H cells. The “C” or “Control” indicates the blank control group, and the “Met” indicates the groups treated with metformin. All the experiments were repeated thrice.

Abbreviations: C, control; Met, metformin; 3-MA, 3-methyladenine.

cells,^{15,21-23} and as described in the section of discussion, the author declared that it was the first report showing that metformin inhibited autophagy in HCC cells.¹⁵

Coincidentally, as shown in Figure 2C–E of our study, we observed that metformin could decrease the expression level of LC3-II in HepG2 cells which was similar to the abovementioned report. However, it was increased in MHCC97H cells, and the p62 expression was decreased both in MHCC97H and HepG2 cells. LC3 is involved in the formation of autophagosomes and p62 is usually used to monitor the autophagic flux. Therefore, we deduced that

metformin might have no obvious effect on the formation of autophagosomes in HepG2 cells, but it could affect the autophagy flux and induce the cell autophagy. In addition, it might be different in different types of HCC cells.

AMPK-mTOR signaling pathway is well known to be associated with autophagy and this pathway has been reported to be the potential mechanism for multiple molecular targets and therapeutic approaches in HCC, for example radiofrequency ablation (RFA) and heat stress, bifunctional enzyme ATIC, glycochenodeoxycholate (GCDC) and SOX18 expression.²⁴⁻²⁷ RFA and heat

stress could induce the autophagy in HCC SMMC7721 and Huh7 cells, and autophagy was induced via the AMPK-mTOR axis.²⁶ Li et al demonstrated that ATIC expression was up-regulated in HCC tissues and HCC patients with high level of ATIC had poor survival. They declared that ATIC was identified as one oncogenic gene that promotes proliferation and migration via the AMPK-mTOR-S6 K1 signaling.²⁴ GCDC and downregulation of SOX18 could also activate the autophagy by targeting the AMPK-mTOR pathway in HCC cells.^{25,27}

Some limitations should be acknowledged in our study. The first was that MHCC97H and HepG2 cell lines were used and the reported experiment results were mostly from the MHCC97H cells considering LC3-II expression was decreased by metformin in HepG2 cells. We hope that these results can be validated in more HCC cell lines. The second was that only some autophagy-related proteins and AMPK-mTOR signaling pathways were examined, and the results were only from in vitro cell culture tests. We wish that more signaling pathways and molecular biomarkers could be verified in future studies, and more experimental methods and research strategies are adopted.

In conclusion, our study found that metformin treatment could induce the autophagy, autophagic flux, and activate the AMPK-mTOR signaling pathway in human HCC cells. We anticipate that combination treatment with metformin and molecular markers of the AMPK-mTOR signaling pathway could be used in HCC therapy.

Ethics Approval

Our research project was approved by the Clinical Research Ethics Committee of China-Japan Friendship hospital (Beijing, China). The human HCC HepG2 cell lines had been authenticated by STR profile and the use was approved by the Clinical Research Ethics Committee of China-Japan Friendship hospital.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest.

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