Combination therapy with metformin and IL-12 to inhibit the growth of hepatic carcinoma by promoting apoptosis and autophagy in HepG2-bearing mice

Z. JIN, B.-X. JIA, L.-D. TAN, Q.-M. CHEN, Y.-H. LIU

Department of Hepatobiliary and Pancreatic Surgery, the First Hospital of Jilin University, Changchun, Jilin, China

Abstract. OBJECTIVE: To investigate the effects and mechanism of metformin (Met) combined with interleukin-12 (IL-12) on inhibiting hepatoma HepG2 cell proliferation via in vitro and in vivo assays.

MATERIALS AND METHODS: MTT assay was used to detect inhibitory effects of Met, IL-12 alone or combination on HepG2 cells proliferation. Half inhibitory concentration (IC50) and combination index (CI) were also calculated. Anti-tumor effects of combination or monotherapy on the HepG2-bearing mice were investigated and protein expression levels of apoptosis, as well as the Akt/mTOR/STAT3 signaling pathway-related factors were detected by Western blot.

RESULTS: MTT results showed that the inhibitory effect of Met combined with IL-12 on HepG2 cell proliferation was significantly enhanced (both p<0.01) compared with monomer therapy group with a significant synergistic effect (CI<1). The apoptosis rate of HepG2 cells treated with Met combined with IL-12 were 88.12±7.15% and significantly higher than the others (all p<0.01). Moreover, combination treatment significantly suppressed hepatoma growth and increased the survival rate of HepG2-bearing mice without evident body weight loss. Western blot analysis showed that met combined with IL-12 significantly increased the expression of autophagy-related proteins, downregulated the protein expression levels of Bcl-2, p-Akt, p-mTOR, p-STAT3, BAX, and upregulated the expression level of BAX in both HepG2 cells and tumor tissues.

CONCLUSIONS: Met combined with IL-12 exhibited a synergistic antitumor effect on hepatoma HepG2 cells, and the mechanism may be related to its common inhibition of Akt/mTOR signaling pathway and increase of autophagy in HepG2-bearing mice.

Key Words: Metformin, Interleukin-12, Combination therapy, HepG2 cell, Apoptosis.

Abbreviations
Human hepatocellular carcinomas (HepG2); Metformin (Met); Interleukin-12 (IL-12); Inhibitory concentration (IC); Combination index (CI); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT); Interferon-γ (IFNγ); Hepatocellular carcinoma (HCC); Polyacrylamide gel electrophoresis (SDS-PAGE); Electrochemiluminescence (ECL); Protein Kinase B (AKT); Mammalian target of rapamycin (mTOR); Signal transducer and activator of transcription 3 (STAT3); BCL2-Associated X (Bax); Hematoxylin and eosin (H&E).

Introduction

In recent years, metformin (Met), as first-line anti-diabetes drug with few side effects, low price and wide clinical application has been widely reported1-4. Met can reduce the incidence of various cancers in diabetic patients, including hepatocellular carcinoma5-6. According previous reports, the anti-tumor mechanism of Met is related to the activation of AMP-activated protein kinase (AMPK) and canonical PI3K/Akt/mTOR signaling pathway. Donadon et al7 found that the use of other oral hypoglycemic agents, such as insulin secretagogues or insulin can increase the incidence of liver cancer, while the use of Met exhibited a reverse trend. This study retrospectively analyzed 618 patients with liver cirrhosis who were orally administered with Met and other hypoglycemic agents, and found that the risk of liver cancer was significantly reduced in the group treated with Met (OR=0.15, 95% CI 0.04~0.50, p<0.001)7. Nkontchou et al8,9 reported that the incidence of liver cancer tended to be lower in the Met group than in the group taking other hypoglycemic agents (HR=0.19, 95% CI 0.04~0.50, p=0.003).
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0.04 to 0.79, \( p<0.05 \)), and also found that Met was associated with a lower incidence of liver-related death. Moreover, the combination of Met with chemotherapy or radiotherapy has a synergistic effect in a variety of tumor cells, and may enhance the radiation-mediated apoptosis and proliferation inhibited the hepatocellular carcinoma and reversed the multidrug resistance of hepatocellular carcinoma cells\(^{10-13} \text{. Bhalla et al}^{14} \text{ found that liver cancer size of Met-treated model mice was 57\% lower than that of the control group indicating that Met has a certain effect on the prevention and treatment of liver cancer.}

IL-12, as an attractive antitumor therapeutic cytokine, can activate the innate and adaptive arms of the immune system and is able to elicit antigen-specific immune responses\(^7\). IL-12 holds potency to promote the polarization of T helper type 1 cells, which leads to secretion of interferon-\(\gamma \) (IFN\(\gamma \)) from effector cells, such as CD8\(^+ \) T cells and stimulation of antigen presentation\(^8\). The anti-tumor effect of IL-12 is not only through immunomodulation of the tumor microenvironment, it can also induce peritumoral angiogenesis, thereby reducing the supply of nutrients and oxygen to tumor cells\(^11\). Moreover, IL-12 is able to inhibit the invasion and reduce the survival time of hepatocellular carcinoma (HCC) cells\(^18\). In addition, IL-12 can also induce significant apoptosis of HCC cells which may act by inhibiting the expression of survivin gene\(^19\). In HCC cells, autophagy can protect the liver but inhibit tumor progression, such as inflammatory response, tissue damage, and cell viability\(^20\). Previous reports confirm that the IFN-\(\gamma \) and TGF-\(\beta \) can induce autophagy in HCC cells which in turn inhibits the proliferation of HCC cells and promotes apoptosis\(^20\). On the other hand, the survival of HCC cells under stress conditions require the involvement of autophagy, and IL-12 can induce autophagy in HCC cells during treatment\(^20\).

There is no report on the combination treatment of the Met and IL-12 on HCC cells, and the mechanism has also not been clarified. Therefore, present research provides new ideas and experimental basis for the treatment of liver cancer by studying the anti-tumor effects of combination treatment on the proliferation, autophagy and apoptosis of HepG2 cells and tumor growth in HepG2-bearing mice.

Materials and Methods

**Materials**

Met was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). IL-12 with purity over 95% and the antibodies targeting β-actin, ERK, p-ERK, Akt, p-Akt, STAT3, mTOR, and so on, were all purchased from R&D Company (Minneapolis, MN, USA). The antibodies targeting Bel-2, BAX and HRP-labeled anti-rabbit secondary antibodies were purchased from Sequoia Jinqiao Biotechnology Company (Beijing, China). Other reagents used in experiments were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

**Cell Strain and Culture**

The hepatoma cell line HepG2 (ATCC® HB-8065™) and normal cell line L-02 (gift from the Molecular Biology Laboratory of Peking University) were stored in an ultra-low temperature ice box at -80°C or liquid nitrogen for a short or long period, respectively. Cell resuscitation and passage were performed every 3 months, and cryopreservation was continued. The cells were cultured in DMEM medium with 10% FBS in a CO\(_2\) incubator with constant temperature (37°C). The culture medium was discarded when the cells grew to 80%, and then the cells were rinse with PBS for 3 times. The trypsin was added with gently shake up until the whole bottom were covered, then suck off the residual trypsin with a pipette, place in the incubator for about 3 min and trypan blue was continued. The cells were cultured in DMEM medium with 10% FBS in a CO\(_2\) incubator with constant temperature (37°C). The culture medium was discarded when the cells grew to 80%, and then the cells were rinse with PBS for 3 times. The trypsin was added with gently shake up until the whole bottom were covered, then suck off the residual trypsin with a pipette, place in the incubator for about 3 min and trypan blue was continued. The cells were cultured in DMEM medium with 10% FBS in a CO\(_2\) incubator with constant temperature (37°C).

**Cell Proliferation Inhibition Test**

Different concentrations of Met or IL-12 were added to each well, containing 1E5 L-02 cells, to final concentrations of 2.5, 5, 10, 20, 40, 80 and 160 mmol/L, respectively, and 0.1% DMSO was used as control. Moreover, the combination incubation of Met and IL-12 was set at the half concentration of each molecules. The HepG2 cells were divided into five different groups for treatment: Met group (10, 20, and 40 mmol/L), IL-12 group (10, 20 and 40 mmol/L), combination incubation (both Met and IL-12 at 5, 10, and 20 mmol/L), and negative control group (cells only, without drug treatment). Six replicate wells were set up for each group. The cells were incubated in a thermostatically saturated humidity incubator
at 37°C with CO2 volume fraction of 5% for 72 h. The cryopreserved 5 mg/ml MTT solution was taken out in advance and melt in the dark at room temperature. After 72-hour incubation, the 96-well plate was added with 20 μL MTT solution in the dark for 4 hours. Then, the cell culture medium was slowly suck off and the 200 μL DMSO solution was added to each well. The absorbance value of the detection at the wavelength of 490 nm in different groups were measured by microplate reader. Calculation of the cell survival rate: (absorbance value sample - absorbance value blank)/(absorbance value control - absorbance value blank) ×100%. The Statistical Product and Service Solution (SPSS) 16.0 software was applied to calculate the half inhibitory concentration (IC50) of Met and IL-12 on HepG2 cells. Moreover, Compu Syn software was used to calculate the combination index (CI) when the two drugs were combined, CI<1 expressed that the two drugs had a synergistic effect, CI=1 indicated that the two drugs had an additive effect, and CI>1 manifested that the two drugs had an antagonistic effect.

Fluorescent Staining
After 24 hours of drug treatment, the supernatant in the 6-well plate was slowly removed, and the cells were rinsed twice with PBS with gentle movements to prevent cell detachment. According to the instructions of Annexin V-FITC Apoptosis Detection Kit, binding solution (500 μl/well) and Annexin V-FITC (5 μL/well) were successively added, gently shaken and mixed, protected from light for 10 to 15 min; finally PI staining solution (10 μl/well) was added, protected from light for 5 min; placed in a fluorescence microscope, a two-color filter was used to observe the morphological degeneration of apoptotic cells, and photographs were taken. PI is a nucleic acid dye that cannot penetrate the intact cell membrane of normal cells, but in cells in the late stage of apoptosis or necrotic cells, PI can penetrate the cell membrane and present red fluorescence in combination with the nucleus, Annexin V can bind the cell membrane in the early stage of apoptosis and present green fluorescence.

Establishment of HepG2 Subcutaneous Xengraft Model
Female BALB/C mice aged 4-6 weeks, weighing 15-20 g, were adaptively reared. Animal experiments in the present study were approved by the Animal Investigation Ethics Committee of the First Hospital of Jilin University and the experimental license number is FHZJLU-20190072. All animal performances were in strict accordance with the International Guideline for the Care and Use of Laboratory Animals. HepG2 cells in logarithmic growth phase were cultured, digested with trypsin, resuspended in PBS, and the concentration of HepG2 cells was adjusted to 2.5×10⁶/mL (about 10⁶ cells contains 5x10⁶ liver cancer cells). The prepared HepG2 cells were injected subcutaneously into the left axilla, with a volume of 0.2 mL/injection, taking care to prevent leakage. Tumor growth was observed every other day about 7-10 days, the subcutaneous tumor were successfully modeled with a volume of about 100 mm³. Thirty mice were randomly divided into four groups: negative control group, IL-12 group (10 μg/mice), Met group (1 mg/mice), Met and IL-12 combination group (5 μg IL-12 and 0.5 mg Met/mice). The health conditions such as activity in the cage, food intake, defecation, and mental state were observed daily, and the body weight was weighed and recorded every 7 days. The size of tumors was measured every 2 days using a digital caliper. The long diameter (indicated by a) and short diameter (indicated by b) were recorded, and the tumor size was calculated. The tumor volume size was calculated according to the formula V = 1/2ab² and the tumor suppression curve was plotted. The formula for tumor inhibition rate was calculated: tumor inhibition rate (%) = (tumor volume in the control group–tumor volume in the treatment group)/tumor volume in the control group × 100%.

At the end of eight weeks of administration, venous blood samples were collected and the supernatant was obtained by centrifugation and stored in a -80°C refrigerator. The fresh hepatocellular carcinoma tissue from tumor-bearing mice were removed and then fixed in 10% formalin after being rinsed with PBS for 3 times and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. Histological examination was completed by using an Olympus microscope (Life Science Solutions, San Jose, CA, USA). In addition, tumors of the tumor-bearing mice were separated and then performed H&E staining according to the standard operating procedures.

Western Blot Analysis
According to different groups, the total proteins of each group was extracted. The concentrations of the proteins were determined according to the BCA method, and each histone was
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Results

Met Combined With IL-12 Exhibited Synergistic Inhibitory Effects on the Proliferation of HepG2 Cells

The cytotoxicity of IL-12 and Met on the viability of L-02 cells, a kind of human normal hepatocytes, was assessed through the MTT method. As shown in the Figure 1, continuously increased concentrations of Met or IL-12 within the range of 2.5 to 40 mmol/L both did not have a significant effect on the viability of L-02 cells compared with the control. Moreover, the MTT results in HepG2 cells showed that different concentrations of Met and IL-12 exhibited inhibitory effects on the proliferation of HepG2 cells, and the proliferation inhibition rates increased with the increase of drug concentration with the IC50 of Met and IL-12 for 72 h were 37.3±3.5 mmol/L and 21.5±2.3 mmol/L, respectively. Furthermore, combination of IL-12 and Met treatment exhibited more significantly decreased viability of HepG2 cells compared with monotherapy by Met or IL-12 groups at the double final concentration (both \( p < 0.05 \)).

After the growth inhibitory effect of the combination of Met and IL-12 at the corresponding

Figure 1. The protective efficacies of Met combined IL-12 treatment at different doses on L-02 cell viability (A) and HepG2 cell apoptosis (B). \( p < 0.05, 0.01, 0.001 \) using one-way ANOVA (*) and (**, ***). (C) Comparison the effects of Met combined IL-12 with Met or IL-12 alone on cell viability of HepG2 cells. All data were presented as mean ± SD (n=6).
concentrations on HepG2 cells were investigated using MTT assay, we further calculated the CI value when each concentration of the drug was combined. The results in Figure 2 showed that the CI of Met and IL-12 at 2.5, 5, 10, 20 and 40 mmol/L is 0.61, 0.81, 0.72, 0.59 and 0.31, respectively, and the results are all less than 1, suggesting that Met has a synergistic effect with IL-12 on inhibiting the proliferation of HepG2 cells.

**Morphological Changes of HepG2 Cells Apoptosis in Each Group Under Fluorescence Microscope**

As shown in Figure 3, we found significant early apoptosis (green fluorescence), significantly decreased number of adherent cells, and increase intercellular space compared with the negative control group. Compared with the respective incubation group, the HepG2 cells with the same total concentration of Met and IL-12 incubation group showed more significant apoptosis, increased densely stained red fluorescence (late apoptosis) in the nucleus or cytoplasm, remarkably reduced number of adherent cells. Moreover, the intercellular space was significantly increased, the cells became round, showing evident apoptotic characteristics, and the HepG2 cells treated with combination of Met and IL-12.

**Combination of Met and IL-12 Increase the Expression of Apoptosis-Related Proteins in HepG2 Cells**

We further investigated the effects of Met and IL-12 and combination treatment on HepG2 apoptosis-related proteins. The Western blot results in Figure 4 showed that Met and IL-12 could decrease the expression of p-Erk protein,
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$p$-Akt protein and anti-apoptotic protein, Bcl-2, and increase the expression of pro-apoptotic protein, Bax. Compared with the monotherapy group, the combined incubation of Met and IL-12 exhibited a more significant inhibitory effect on the upregulation of pro-apoptotic proteins and anti-apoptotic proteins, and there were statistically significant differences (all $p < 0.01$) showing that the combination treatment could effectively promote the apoptosis of HepG2 cells by upregulating the expression of pro-apoptotic proteins.

**Combination of Met and IL-12 Suppressed the AKT/mTOR/STAT3 Signaling Pathway**

AKT/mTOR is a signaling pathway that inhibits autophagy and is activated during the inhibition of tumor cell proliferation by many drugs. Therefore, we first further examined the activation of this signaling pathway by the combined treatment of Met and IL-12. As shown in Figure 5, the expression of $p$-mTOR and $p$-STAT3 (Ser727) in HepG2 cells treated with the Met combined with IL-12 were significantly upregu-
lated compared with the negative control group and the respective monotherapy. The above findings suggested that the induction of autophagy in HepG2 cells by the combined incubation of Met and IL-12 was through the AKT/mTOR/STAT3 signaling pathway.

**Combination of Met and IL-12 Effectively Suppressed the Growth of HepG2-Transplanted Tumor in Mice**

After 7-10 days inoculation, the subcutaneous nodules gradually formed and exceeded 100 mm³, which were considered as successfully established hepatic carcinoma in mice. As shown in Figure 6A, with the growth and proliferation of tumors, the body weight of the mice in each group continuously increased within first four weeks while the those of the mice in negative control group increased slowly after the fourth week which decreased continuously with the increase of tumor-bearing volume.

As shown in Figure 6B, the growth of hepatic carcinoma in mice were significantly inhibited by combination of Met and IL-12 with the inhibitory rate of 93.5% compared with that of the negative control group \( (p<0.001) \). Moreover, the inhibitory rate of the combination group was also significantly higher than that of the monotherapy groups treated with Met (41.5%) or IL-12 \( (p<0.01 \text{ or } p<0.05) \), respectively. Furthermore, as showed in the Figure 6C, combination treatment group exhibited a 100% survival rate while that of the negative control group, Met and IL-12 monotherapy group was 16.7%, 66.7% and 83.3%, respectively. The normal liver removed the tumor tissues were fixed in 10% formaldehyde and then cut into 5 mm slices following by H&E staining to observe the nodules in the liver tissue. As showed in Figure 6D, the tumor tissues of combination treatment group was significantly lower than those of the negative control group. Moreover, the combination of Met and IL-12 also significantly inhibited the formation of liver lesions compared with Met and IL-12 monotherapy group.

Moreover, we detected the expression of autophagy marker protein, LC3, as well as autophagy phase related protein, Beclin1, in hepatoma

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**Figure 6.** Effective suppression of hepatic carcinoma growth in HepG2-bearing mice. The (A) body weight, (B) tumour size, (C) survival individual and (D) Pathological images of the tumour tissues of HepG2-bearing mice were investigated \((×200)\), \( p < 0.05, 0.01, 0.001 \) using one-way ANOVA \((*, **, ***). All results were showed as means ± SD \((n=6)\).
tissues. The Western blot analysis in Figure 7 suggested that the protein expression of LC3-II and Beclin1 were significantly increased in the tumor tissues from the mice treated with combined Met with IL-12 compared with those of the monotherapy and negative control groups. We further detected the expression of apoptosis and Akt/mTOR/STAT3 signaling pathway related proteins in tumor tissues. As shown in Figure 8, the combination treatment could effectively upregulate the pro-apoptotic protein Bax, downregulate the anti-apoptotic protein Bcl-2 and suppress the AKT/mTOR/STAT3 signaling pathway, which were consistent with the in vitro results.

Discussion

Hepatocellular carcinoma (HCC) has become one of the most common malignant tumors due to its malignancy and easy metastasis, and its incidence rate is also increasing at a rate of 3% per year\(^2\)\(^1\). Met is a lipophilic biguanide which could inhibit gluconeogenesis in the liver and improve peripheral utilization of glucose\(^2\)\(^2\). It is currently the first line of pharmacotherapy for glucose control in patients with type 2 diabetes\(^1\). Met also plays an anti-tumor metabolic role through the selective regulatory function of microRNAs (miRNAs) and regulate AMPK-dependent or AMPK-independent pathways\(^2\)\(^3\).

Interleukin 12 (IL-12), as a member of the chemokine family, is an immunomodulatory cytokine composed of two subunits, p35 (35 kDa) and p40 (40 kDa), connected by disulfide bonds, and has become one of the most effective cytokines for potential anti-tumor immunotherapy\(^1\)\(^6\). The antitumor effect of IL-12 is not only through immunomodulation of the tumor microenvironment, it can also inhibit tumour angiogenesis, thereby reducing the supply of nutrients and oxygen to tumor cells; it can directly suppress tumour cells\(^17\). IL-12 is also used to inhibit the invasion and metastasis of HCC cells\(^17\). In addition, IL-12 can also achieve significant apoptosis of tumour cells, which may act by inhibiting the expression of survivin gene\(^19\).

However, the inhibitory effect of Met and IL-12 combination on hepatoma cells has not yet been reported in the literature. Therefore, this study focused on whether combination of IL-12 and Met can synergistically induce hepatoma cell apoptosis, and its probable mechanisms.

In this study, effects of combination treatment of Met and IL-12 on the proliferation of hepatoma cells was examined by MTT assay. The results of cell survival assay in Figure 1, we showed that Met and IL-12 exerted increased cell proliferation inhibition with the increase of drug concentration, and the inhibitory effect...
was more evident in the combined incubation group. By examining the proliferation inhibition rate when the Met and IL-12 were combined, these results were further used to calculate the CI which were all less than 1 at different doses, suggesting that the combination of Met and Met indeed exhibited a significant synergistic effect (Figure 2).

Apoptosis is an autonomously occurring programmed death and most anti-tumor drugs could induce the apoptosis of tumor cells. As showed in Figure 3, the morphological changes of HepG2 apoptosis were more evident in the combination group treated with both Met and IL-12 than those of negative control or monotherapy groups. These changes of apoptosis mainly include nuclear pyknosis, cytoplasmic condensation, cell membrane invagination and apoptotic body formation. The regulatory mechanism of apoptosis is extremely complex, its most common apoptosis-related proteins Bcl-2 (anti-apoptotic protein) and Bax (pro-apoptotic protein) which are both important for the occurrence and development of tumors bind to the mitochondrial membrane, and these two Bax proteins polymerize with each other to form homodimers that promote apoptosis. To further explore the mechanism of pro-apoptotic effects, the expression levels of related signaling pathway proteins and apoptosis-related proteins were detected by Western blot method. As the results showed in Figure 4, both Met and IL-12 could reduce the expression of p-Akt and p-Akt, downregulate the expression of anti-apoptotic protein Bcl-2, upregulate the expression of pro-apoptotic protein Bax, and the above effects were more significant in the combination incubation group. Yang et al. found that Met significantly inhibit the proliferation of HepG2 and BEL7402 cell lines in vitro by inhibiting the expression of Bcl-2 gene, and then, play an anti-tumor role, which was consistent with the results of this study.

In hepatoma cells, autophagy can protect the liver by inhibiting tumor progression, such as inflammatory response, tissue damage, and genomic stability. It has been confirmed that the autophagy in HCC cells in turn inhibited the proliferation of HCC cells and promotes apoptosis. On the other hand, the survival of HCC cells under stress conditions requires the involvement of autophagy, and sorafenib can induce autophagy in HCC cells during the treatment of HCC, but it can significantly enhance the killing effect of sorafenib on HCC cells after silencing autophagy-related genes by siRNA or inhibiting autophagy using autophagy inhibitors. We further found that both Met and IL-12 both slightly decreased the expression of p-Akt and p-Akt, and the inhibitory effects were more significant after combined incubation. Moreover, Akt/mTOR/STAT3 is a signaling pathway that inhibits autophagy and is activated during the inhibition of tumor cell proliferation by many drugs. Therefore, we first further examined the activation of this signaling pathway in tumor tissues (Figure 8).

Figure 8. The effects of Met combined IL-12 treatment on apoptosis-related proteins and Akt/mTOR/STAT3 signaling pathway in tumor tissues. (A) Western blot analysis of the protein expression of (B) Bcl2, (C) BAX, (D) p-Akt, (E) Akt, (F) p-mTOR, (G) t-mTOR, (H) p-STAT3 and (I) t-STAT3. p<0.05, 0.01 using one-way ANOVA (*, **) and (*) vs. Met and IL-12 group, respectively. Results were showed as means ± SD (n = 6 each group).
pathway by the combined treatment of Met and IL-12. The protein expression level of p-mTOR and p-STAT3 showed in Figure 5 proved that combination treatment significantly downregulated compared with the negative control group or others. The above data showed that the induction of autophagy in HepG2 cells by the combined incubation group of Met and IL-12 was through the AKT/mTOR/STAT3 signaling pathway.

After the HepG2-bearing mice were successfully established, the chronic treatment of Met combined with IL-12 were performed. As the results showed in Figures 6A-B, body weight of the mice in all four groups continuously increased within first four weeks as well as the tumor size. However, the body weight of tumor-bearing mice in negative control increased slowly after the fourth week, which was even lower at the week 8 than at the week 1. As showed in Figure 6B, the tumor growth of the tumor-bearing model mice were significantly inhibited by combination of Met and IL-12 with the inhibitory rate of 93.5% which was significantly better than all other groups (all p<0.05). Furthermore, combination treatment group exhibited a 100% survival rate compared to that of the negative control group, Met or IL-12 monotherapy group was 16.7%, 66.7% or 83.3% respectively (Figure 6C). Further H&E staining results of tumors were showed in Figure 6D, the nodules and formation of liver lesions in the tissues of combination treatment group were significantly lower than the control, Met group or other groups.

Autophagy, as a ubiquitous stress metabolic process in the cells, is required in maintaining the normal physiological balance of the cell and the stability of the internal environment, are closely related to the development of tumors. Traditional method for the treatment of cancer is to induce the apoptosis of tumor cells. However, the emergence of apoptosis resistance in tumor has become a major obstacle to cancer treatment. Recently, tumor cell death induced by other cell death mode has become a potential new anti-cancer mechanism. Autophagy is a programed cell death mode that involves the complex molecular mechanisms as autophagy, and there are close connections between them, such as many similar proteins. According to the published reports, autophagy is required for apoptosis which usually initiates the programed cell death mode that has the same complex molecular mechanisms as apoptosis and there are close connections between them, such as many similar proteins. According to the published reports, autophagy is required for apoptosis which usually initiates autophagy of tumor cells which may also be the one of the reasons for the synergistic effect.

In summary, Met combined with IL-12 had a synergistic anti-tumor effect on HepG2 cells in vitro and in vivo by inhibiting cell proliferation and promoting apoptosis. The combined effect was significantly stronger than that of Met or IL-12 alone, which may be related to its common inhibition of Akt/mTOR/STAT3 signaling pathway by the combined treatment of Met and IL-12. The protein expression level of p-mTOR and p-STAT3 showed in Figure 5 proved that combination treatment significantly downregulated compared with the negative control group or others. The above data showed that the induction of autophagy in HepG2 cells by the combined incubation group of Met and IL-12 was through the AKT/mTOR/STAT3 signaling pathway.

Conclusions

Metformin plus IL-12 inhibits HCC growth in vitro and in vivo by inhibiting cell proliferation and promoting apoptosis. The combined effect was significantly stronger than that of Met or IL-12 alone, which may be related to its common inhibition of Akt/mTOR/STAT3 signaling pathway by the combined treatment of Met and IL-12. The protein expression level of p-mTOR and p-STAT3 showed in Figure 5 proved that combination treatment significantly downregulated compared with the negative control group or others. The above data showed that the induction of autophagy in HepG2 cells by the combined incubation group of Met and IL-12 was through the AKT/mTOR/STAT3 signaling pathway.
pathway and induction of autophagy in HCC cells or hepatocellular carcinoma tissues. The novelty of this study is that the combination of Met and IL-12 has a synergistic inhibitory effect on the growth of hepatocellular carcinoma cells for the first time, and its mechanism is explored from multiple aspects, such as autophagy, apoptosis and Akt/mTOR/STAT3 signaling pathway which also provides a pharmacodynamic and theoretical basis for the further application of this drug combination in subsequent clinical practice.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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