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

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

MATERIALS AND MEMBERS: 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. Antitumor effects of combination or monotherapy on HepG2-bearing mice were investigated and protein expression levels of apoptosis, as well as Akt/mTOR/STAT3 signaling pathway-related factors were detected by Western blot.

RESULTS: MTT results showed the inhibitory effect of Met combined with IL-12 on HepG2 cell proliferation was significantly enhanced (both $p<0.01$) compared with monotherapy group with a significant synergistic. The apoptosis rate of HepG2 treated with Met combined with IL-12 were ±7.15% and significantly higher than the other ($p<0.01$). 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 and downregulated the protein expression levels of Bcl-2, p-Akt, p-mTOR, p-STAT3, and equally the expression level of Bax in both HepG2 cells and tumor tissues.

CONCLUSIONS: Met combined with IL-12 exhibits a synergistic antitumor effect on hepatoma cells, and the mechanism may be related to its common inhibition of Akt/mTOR/STAT3 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); Half inhibitory concentration (IC50); Combination index (CI); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT); Interferon-γ (IFN-γ); Hepatocellular carcinoma (HCC); Polyacrylamide gel electrophoresis (SDS-PAGE); Enhanced chemiluminescence (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 reported[1-4]. Met can reduce the incidence of various cancers in diabetic patients, including hepatocellular carcinoma[5,6]. 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 al[7] 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 al[8,9] 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

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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]. Bhalla et al[14] 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[15]. IL-12 holds potency to promote the polarization of T helper type 1 cells, which leads to secretion of interferon-γ (IFNγ) from effector cells, such as $CD8^+$ T cells and stimulation of antigen presentation[16]. The anti-tumor effect of IL-12 is not only through immunomodulation of the tumor microenvironment, it can also inhibit peritumoral angiogenesis, thereby reducing supply of nutrients and oxygen to tumor cells[17]. 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, autophagy can protect the liver by inhibiting tumor progression, such as inflammatory response, tissue damage, and genomic stability[20]. Previous reports confirm that both IFN-γ and TGF-β can induce autophagy in HCC cells which in turn inhibit the proliferation of HCC cells and promote apoptosis[20]. On the other hand, the survival of HCC cells under stress conditions requires the involvement of autophagy, and IL-12 can promote autophagy in HCC cells during treatment[21].

To the present date is no report on the combination treatment of the Met and IL-12 on HCC 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 95% and the antibodies targeting β-actin, LC3, p-Erk, Akt, p-Akt, STAT3, mTOR and so on, were all purchased from R&D Company (Minneapolis, MN, USA). The antibodies targeting Bcl-2, BAX and HRP-labeled goat anti-mouse and anti-rabbit secondary antibodies were purchased from Sequoia Jinqiao Biotechnology Company (Beijing, China). Other commonly used reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Cell Strain and Cell 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 freezer 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 cell 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 after the cells are fully digested by trypsin. We furtherly adjusted the cell density to 1×105 cells/mL, and then uniformly inoculate 100 μL of cells in a 96-well plate for the further experiments.

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 CO_2 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 st tant in the 6-well plate was slowly remove d the cells were rinsed twice with PBS with g movements to prevent cell detachment. Accord to the instructions of Annexin V Apoptos Detection Kit, binding soluti 500 ell) and Annexin V-FITC (5 μL/w were s essively added, gently shaken and d, p light for 10 to 15 min ally ng sol n (10 μl/well) was a , protec om light for 5 min; placed u fluorescen roscope, a two-color f wa d to obse the mor- phological egeneration apoptotic cells, and photogra were taken. nucleic acid dye that c t pene rate the inta ell membrane of nor cells, h cells in the late stage of apoptosis ne ic cells PI can penetrate the cell membr d prese d fluorescence in combi- n wi s. Annexin V can bind the ce embra e early stage of apoptosis and pr t green fl rescence.

Establishment of HepG2 Subcutaneous Xenograft Model

e female BALB/C mice aged 4-6 weeks, we ning 15-20 g, were adaptively reared. Animal experiments in the present study were approved by the Animal Investigation Ethic Committee of the First Hospital of Jilin University and the experimental license number is FHJ-LU-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 ph cultured, digested with trypsin, res ded PBS, and the concentration of h oma cells was adjusted to 2.5×10^7/ml (about contains 5×10^6 liver cancer cells). Th prep HepG2 cells were injected subcut usly int left axilla, with a volume 2 ml per inj taking care to preven akage nor gro was observed every ays er abo 7-10 days, the subcuta s t ere su sfully modeled with lume of tha 0 mm^3. Thirty mice randomly into four groups: n ve ol group, 12 group (10 μg/mice), Met group g/mice), Met and IL-12 comb group (5 12 and 0.5 mg Met/ m The health conditi s such as activity in t age, food intake, defecation, and mental sta- t ere observe ily, and the body weight was w ed and rec ed every 7 days. The size of tum dules measured every 2 days using a digi . The long diameter (indicated by a) and short diameter (indicated by b) were ad, and the tumor size was calculated. The olume size was calculated according to the formula $V = 1/2ab^2$ 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

quantified and equally loaded for SDS-PAGE. At the end of electrophoresis, after membrane transfer and blocking with 50 g/L of skimmed milk powder, rabbit anti-Beclin 1 antibody (1:2000), rabbit anti-p-AKT antibody (1:4000), rabbit anti-Bax antibody (1:2000), rabbit anti-p-Akt antibody (1:3000) and rabbit anti-Bcl-2 antibody (1:4000) were added, respectively. Rabbit anti-β-actin antibody (1:2000) was incubated overnight at 4°C. After washing three times with TBST, and horseradish peroxidase-labeled goat anti-rabbit IgG or horseradish peroxidase-labeled goat anti-mouse IgG (1:5000) was added and incubated at room temperature for 1 h. Membranes were washed, and enhanced chemiluminescence (ECL) was illuminated, photographed and stored.

Statistical Analysis

SPSS 16.0 software was used to analyze the experimental data, and the results were expressed as Mean ± SD. Analysis of variance was used for comparison between multiple groups, and $p<0.05$ was considered statistically si[illegible] cant. Each experiment in this study was [illegible] ed three times.

Results

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

The cytotoxicity of IL-12 and M[illegible] viability of L-02 cells, a kind o[illegible] man normal hepatocytes, was assessed th[illegible] the MTT method. As shown in the Fig[illegible]re 1, c[illegible]ously increased concentrations o[illegible]12 or M[illegible]hin the range of 2.5 to 40 mm[illegible]L both did not a significant effect on [illegible] viabili[illegible] L-02 c[illegible] compared with the co[illegible] M[illegible]ver, th[illegible] MTT results in HepG2 [illegible] sh[illegible]at diff[illegible] concentrations of M[illegible]nd IL-12[illegible]bite[illegible]nhibitory effects on th[illegible]feration o[illegible]na HepG2 cells, and [illegible] p[illegible]ation inhi[illegible]on rates increased with the in[illegible] of drug concentration with IC_{50} of Met [illegible]L-12 for 72 h were 37[illegible].5 mmol/L and 21.[illegible]2.3 mmol/L, respectively. Furthermore, combination of IL-12 and [illegible] treatment e[illegible]bited more significantly decreased viability [illegible] HepG2 cells compared with mo[illegible] trea[illegible]t groups at the double final concen[illegible]oth $p<0.05$).

After the growth inhibitory effect of the com[illegible]n of Met and IL-12 at the corresponding

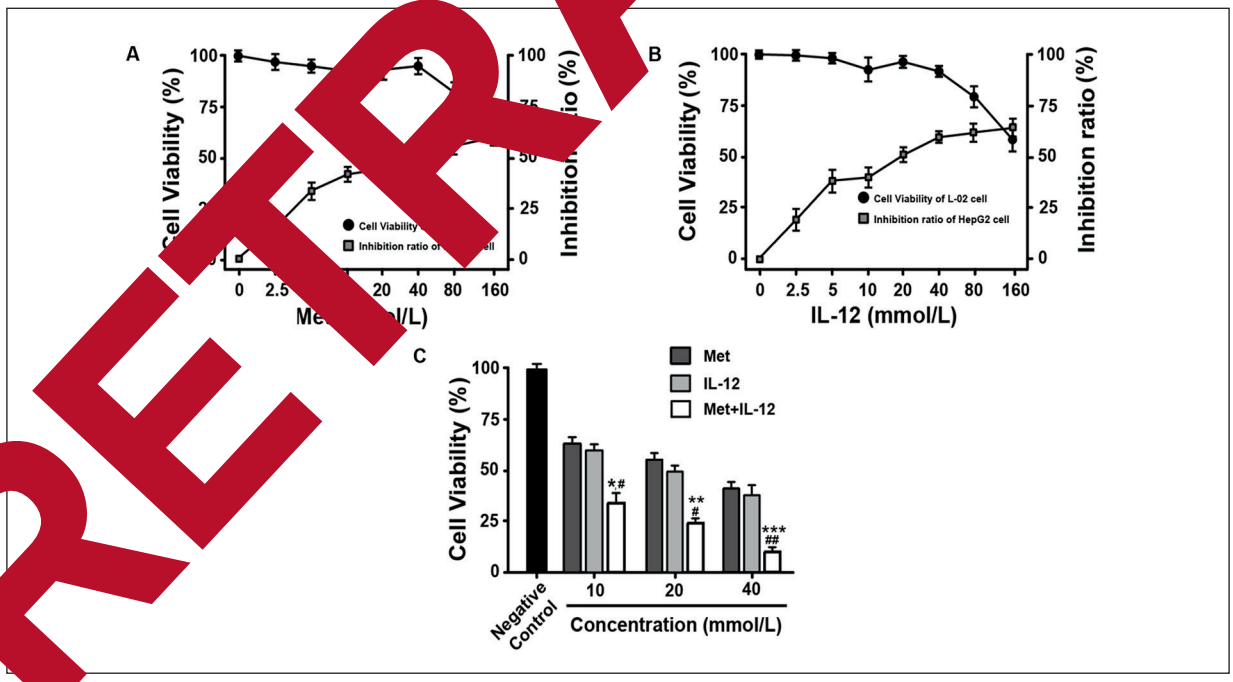

Figure 1. The protective efficacies of Met combined IL-12 treatment at different doses on L-02 cell viability (**A**) and HepG2 cell apoptosis (**B**). $p < 0.05$, 0.01, 0.001 using one-way ANOVA (*, **, ***) and ($^{\#}$, $^{\#\#}$, $^{\#\#\#}$) *vs.* Met and IL-12 group, respectively. (**C**) Comparison 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).

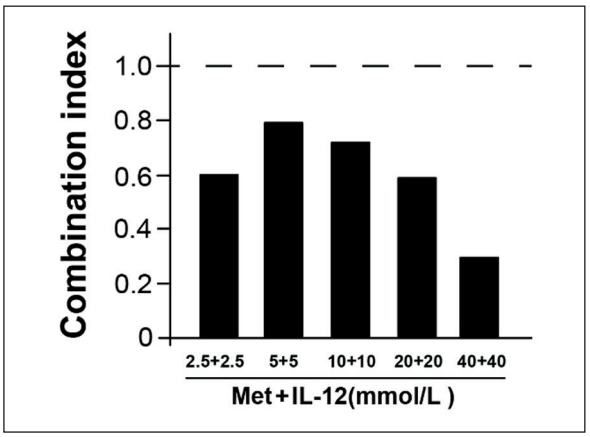

Figure 2. The CI values of Met and IL-12 on the proliferation of HepG2 cells which were analyzed by CompuSyn software.

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 inhibiting the proliferation of HepG2 cells.

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

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

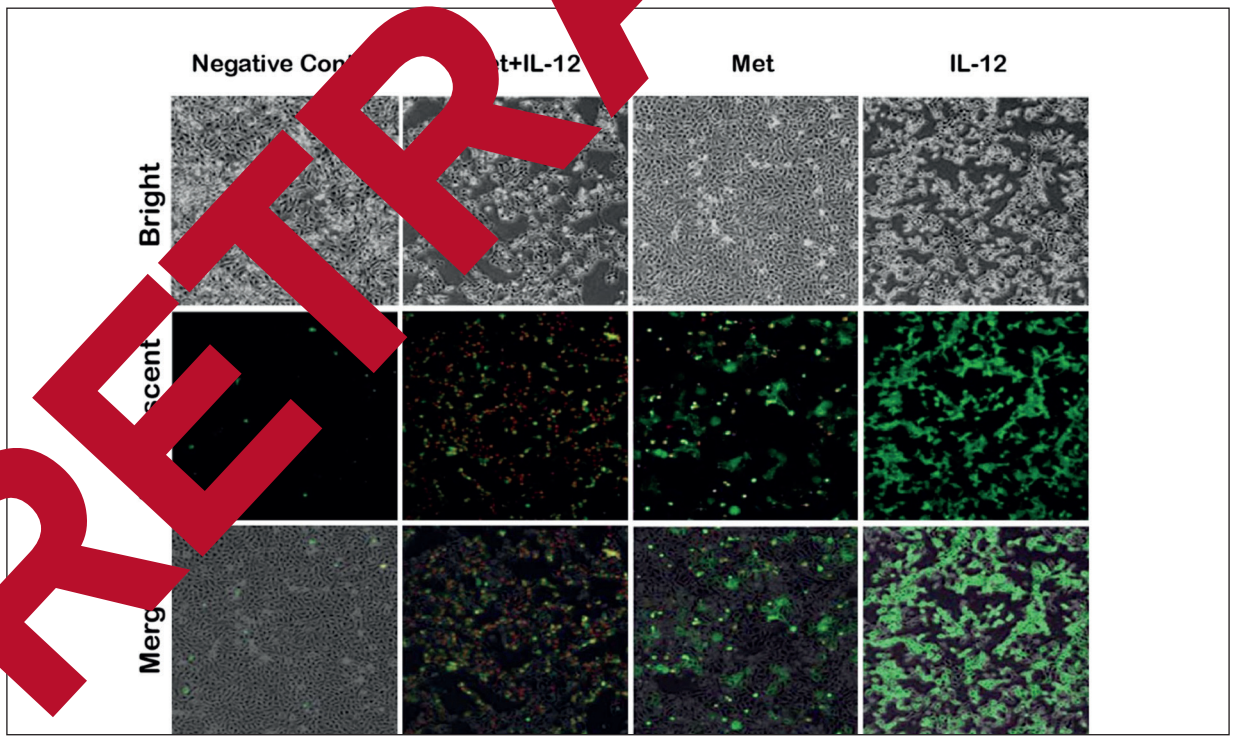

Figure 3. Morphological changes of HepG2 cell apoptosis in Met, IL-12 monotherapy and combination group under fluorescence microscope (×400).

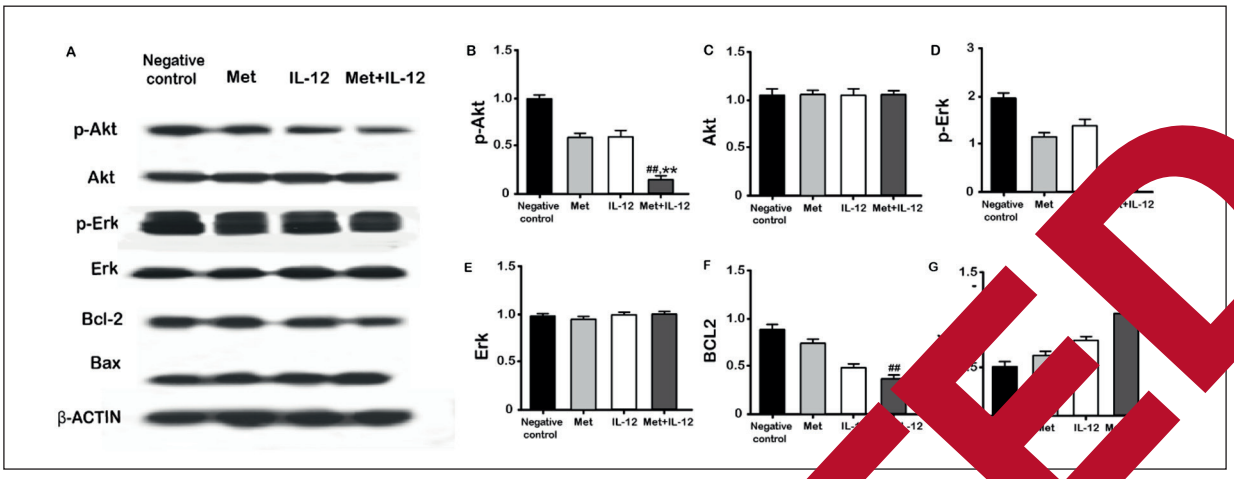

Figure 4. The effects of Met combined IL-12 treatment on apoptosis-related prot pG2 cells. (**A**) blot analysis of the protein expression of (**B**) *p*-Akt, (**C**) Akt, (**D**) *p*-Erk, (**E**) Erk, (**F**) Bcl2 a B 0.05, 0.01, 0. using one-way ANOVA ($^{\#}$, $^{\#\#}$, $^{\#\#\#}$) and (*, **, ***) *vs.* Met and IL-12 group, respectively. Results were showe eans ± SD (n = 6 each group).

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 inhib effect on the upregulation of pro-apopto tein and anti-apoptotic proteins, and there re statistically significant differences (all $p <$ showing that the combination treatment co effectively promote the apopto pG2 cel by upregulating the expres of p poptotic proteins.

mbination of Met and IL-12 pressed th AKT/mTOR/STAT3 Si aling Pat ay

PI3K/ R is a signaling pathway that inhibits gy and is activated during the inhibition of tumor cell proliferation by many Therefore, we first further examined the n 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-

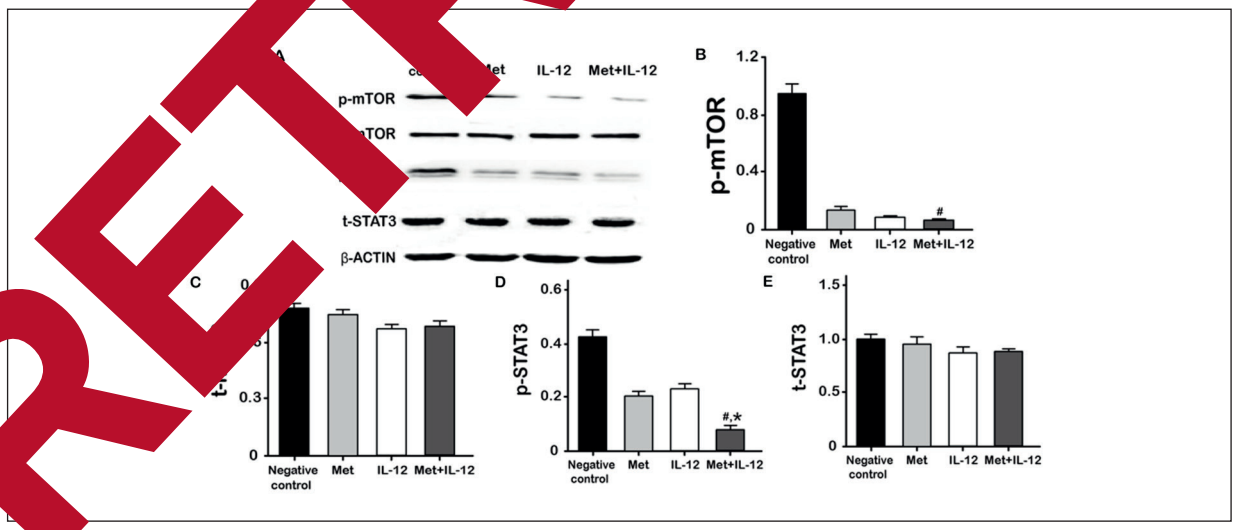

Figure 5. The effects of Met combined IL-12 treatment on AKT/mTOR/STAT3 signaling pathway in HepG2 cells. (**A**) Western blot analysis of the protein expression of (**B**) *p*-mTOR, (**C**) t-mTOR, (**D**) *p*-STAT3 and (**E**) t-STAT3. $p < 0.05$, 0.01, 0.001 using one-way ANOVA ($^{\#}$, $^{\#\#}$, $^{\#\#\#}$) and (*, **, ***) *vs.* Met and IL-12 group, respectively. Results were showed as means ± SD (n = 6 each group).

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 mm^3, 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 or p<0.05, respectively). F as showed in the Figure 6C, com ion treatment group exhibited a 100% sur ate while that of the negative control g up, M IL-12 monotherapy group was 16 66.7% respectively. The norma er removed t mor tissues were fixed 0% fo dehyde then cut into 5 mm th es wing b H&E staining to obser e n the li issue. As showed in F e 6D, the les e tissue of combinati tment grou ignificantly lower t no he negative ontrol group. Moreover, the com ion of Met and IL-12 also antly inhib he formation of liver le compared with M and IL-12 monotherapy group.

Moreover, we tected the expression of autophagy marker tein, LC3, as well as autophagy rel protein, Beclin1, in hepatoma

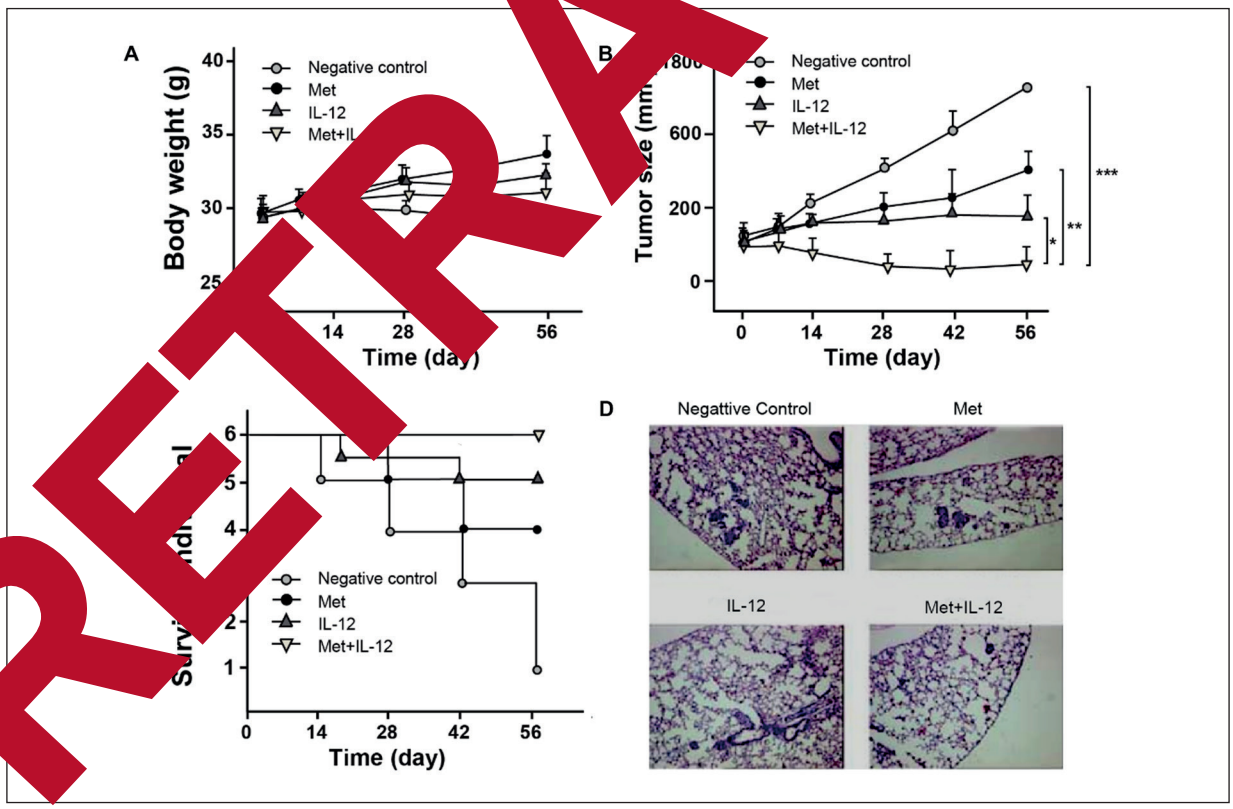

Figure 6. Effective suppression of hepatic carcinoma growth in HepG2-bearing mice. The (**A**) body weight, (**B**) tumour size, (**C**) survival individual and (**D**) Pathological images of the tumour tissues of HepG2-bearing mice were investigated (×200). p < 0.05, 0.01, 0.001 using one-way ANOVA (*, **, ***). 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[21].

Met is a lipophilic biguanide which cou[ld] hibit gluconeogenesis in the liver and impr[ove] ripheral utilization of glucose[22]. It is curren[tly] first line of pharmacotherapy for glucose co[ntrol] in patients with type 2 diabetes[1]. Met also pl[ays] an anti-tumor metabolic role th[rough] selecti[ve] regulatory function of mi[croRNAs] iRNAs) and regulate AMPK-dependent or AMPK-independent pathways[23].

Interleukin 12 (IL-12), as a member of the chemokine family, is an immunomodulatory cytokine composed of two subunits, p35 (3[5] and p40 (40 kDa), connected by disul[fide] bond and has become one of the most e[ffect]ive cytokines for potential anti-tumor im[mu]therapy[16]. The antitumor effect of IL-12 is not e[xert]ed through immunomodulation of the [tu]or micr[oen]vironment, it can also inhibit [the] tumoral angi[o]sis, thereby reducing t[he] supply [of] nutrients [and] oxygen to tumor cells[,] act[ing] ectly on tumor cells[17]. IL-12 is p[ro]d t[o] e to i[nhi]bit the invasion and p[ro]g the s[ur]al ti[me] of HCC cells[18]. In ad[di]tion, IL-12 can a[lso] uce significant apop[to]s[is] o[f] C cells, wh[ich] may act by inhibiting the expre[ssion] of survivin gene[19].

H[owever], the inhibi[tory] ffect of Met and IL-12 c[om]bination on hepa[toma] cells has not yet b[een] reported in the literature. Therefore, this s[tudy] focused o[n] whether combination of IL-12 a[nd] Met can s[yn]gistically induce hepatoma cel[l] [apop]tosis and its probable mechanisms.

In t[his] study, effects of combination treatment of Met and IL-12 on the proliferation of [hepato]ma cells was examined by MTT assay. [Result]s 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

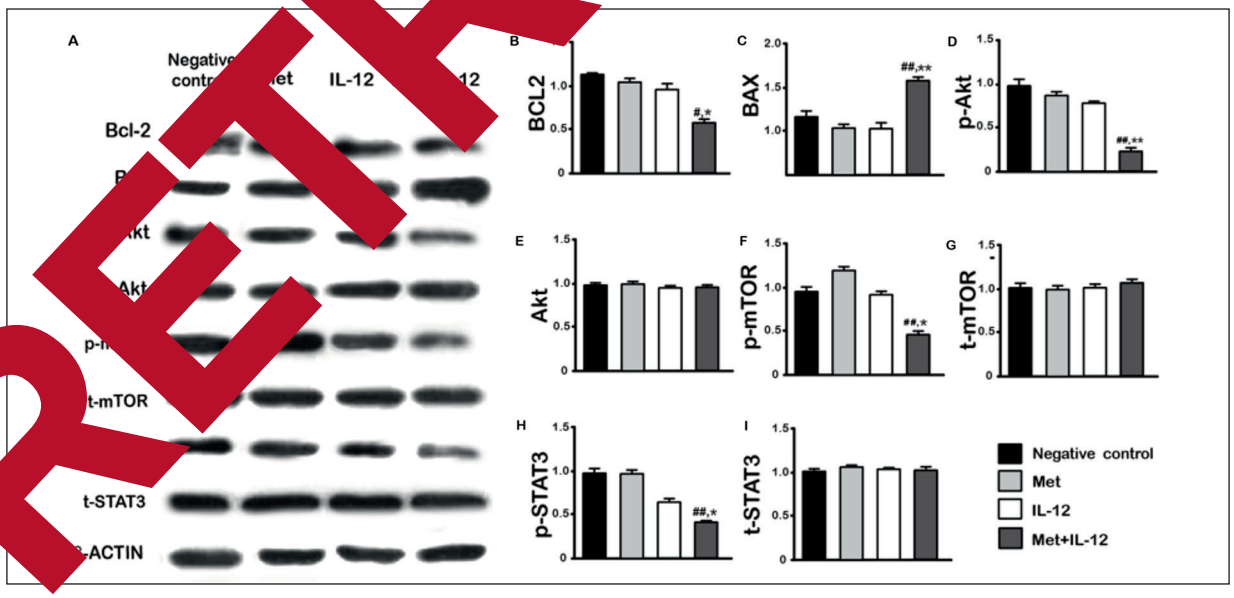

Figure 7. The effects of Met, IL-12 monomer and combination treatment on autophagy-related proteins in tumor tissues. (**A**) Western blot analysis of the protein expression of (**B**) LC3 I, (**C**) LC3 II and (**D**) Beclin-1. $p < 0.05$, 0.01, 0.001 using one-way ANOVA ($^{\#}$, $^{\#\#}$, $^{\#\#\#}$) and (*, **, ***) *vs.* Met and IL-12 group, respectively. Results were showed as means ± SD (n = 6 each group).

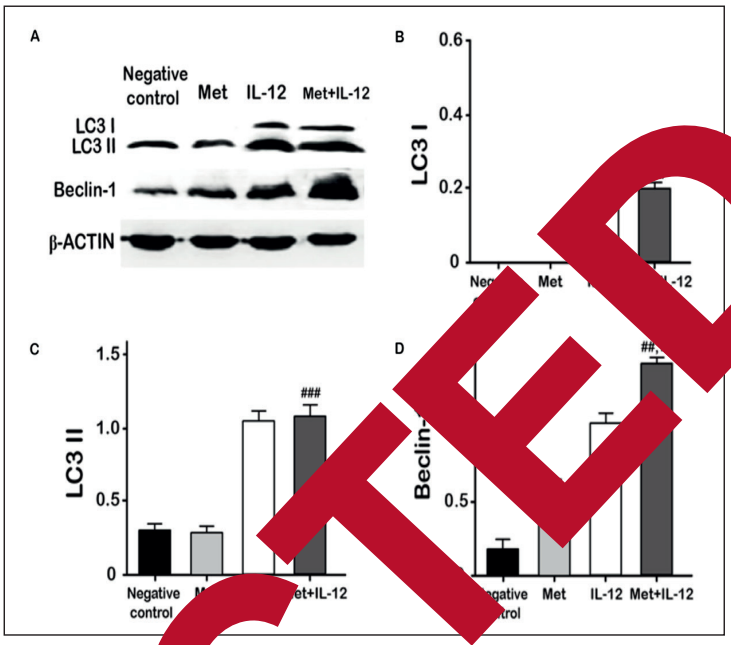

Figure 8. The effects of Met combined IL-12 treatment on apoptosis-related proteins and Akt/mTOR/STAT3 signaling pathway in tumor tissues. (**A**) Western blot analysis of the protein expression of (**B**) Bcl2, (**C**) BAX, (**D**) p-Akt, (**E**) Akt, (**F**) p-mTOR, (**G**) t-mTOR, (**H**) p-STAT3 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).

was more evident in the combined incubation group. By examining the proliferation inhibition rate when the Met and IL-12 were comb these results were further used to calcula the CI which were all less than 1 at dif t doses, suggesting that the combination of I and Met indeed exhibited a significant synerg tic effect (Figure 2).

Apoptosis is an autonom oc ing pro- grammed death and most tumor gs could induce the apoptosis of t cell in Figure 3, the morp gic s of Hep 2 apoptosis were m vident i combination group treated wi Met and I han those of negative co o therapy gr ps. These changes of poptosis ma include nuclear pyk- nosis, c asmic conden cell membrane invagi n and apoptotic b y formation. The reg ry me ism of apoptosis is extremely com a ts mo common apoptosis-re- lated p are B (anti-apoptotic protein) Bax (p c protein) which are both im ant fo occurrence and development of ors. Bax nds to the mitochondrial mem- l two Bax proteins polymerize with ch other to form homodimers that promote ptosis. To further explore the mechanism of poptotic effects, the expression levels of re d signaling pathway proteins and apopto- sis-related proteins were detected by Western blot method. As the results showed in Figure 4, both Met and IL-12 could reduce the expression o rk and *p*-A downregulate the expression of protein Bcl-2, upregulate the expres o-apoptotic protein Bax, and the bove effects were more significant in the combi- treated group. Yang et al[24] found that Met u gnificantly inhibit the proliferation of HepG2 and BEL7402 cell lines *in vitro* by inhib- iting 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 ge- nomic stability[20]. It has been confirmed that the autophagy in HCC cells in turn inhibited the pro- liferation of HCC cells and promotes apoptosis[20]. On the other hand, the survival of HCC cells un- der 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 autopha- gy-related genes by siRNA or inhibiting autopha- gy using autophagy inhibitors[25]. We further found that both Met and IL-12 both slightly decreased the expression of *p*-Erk and *p*-Akt, and the inhibi- tory 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 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 treat[illegible]t group exhibited a 100% survival rate[illegible] that of the negative control group, Met or [illegible]2 monotherapy group was 16.7%, 66.7% or 83[illegible] respectively (Figure 6C). Further H&E stain[illegible] results of tumors were showed [illegible]e 6D, t[illegible] nodules and formation of li[illegible]sio[illegible] the tissues of combination trea[illegible]t grou[illegible]ere significantly lower than tha[illegible] ne[illegible] group or other group[illegible]

Autophagy, as [illegible]iquitous[illegible]s metabolic process in the [illegible]nd cells, [illegible]tains cell homeostasis [illegible]deg[illegible]g some ab[illegible]rmal organelles a[illegible] is vital fo[illegible] survival of normal or tumo[illegible]s. As Western [illegible] analysis showed in Fig[illegible]7, the protein ex[illegible]ssion of LC3-II and [illegible]lin1 w[illegible] significantly increased in the grou[illegible]at [illegible]with M[illegible] combined with IL-12 compa[illegible]h the [illegible]notherapy and negative [illegible]ol gr[illegible]in[illegible]ing the increased autophag[illegible] comb[illegible] therapy group. We further de[illegible]ed the ex[illegible]ression of apoptosis and Akt/[illegible] signaling pathway related proteins in the tumor tissues from HepG2-bearing [illegible]ce. As the results showed in the Figure 8, [illegible]ombination treatment of Met and IL-12 effe[illegible]tively upregulated the Bax, downregulated the Bcl-2 and AKT/mTOR/STAT3 signaling pathway, which were consistent with the *in vitro* results in HepG2 cells. The above findings indicated that AKT/mTOR/STAT3 signaling pathway was involved in autophagy induced by Met and IL-12 co-incubation in HCC model.

It is worth discussing that apoptosis and autophagy, as important mechanisms inv[illegible] maintaining the normal physiologi[illegible] of the machine and the stability [illegible]e internal environment, are closely related [illegible]e develop[illegible]ment of tumors. Traditional met[illegible]for the treatment of cancer is to in[illegible] the ap[illegible] of tumor cells. However, th[illegible]ergence of ap[illegible] ic resistance in tumor[illegible]s has [illegible]me a m[illegible] obstacle to cancer t[illegible]ent. [illegible]cently, tumor cell death induce[illegible] ot[illegible] death[illegible]e has become a pote[illegible] new an[illegible]or [illegible]hanism. Autophagy [illegible]her progra[illegible] cell death mode tha[illegible]as [illegible]ame comp[illegible]x molecular mechanism and reg[illegible]y mechanism as apoptosis. [illegible]here are c[illegible]onnections between th[illegible] such as many simi[illegible] proteins. According t[illegible]e published r[illegible]ports, autophagy is required f[illegible]apoptosis w[illegible] usually initiates the progr[illegible] of apoptosis. [illegible] breast cancer cell line MCF-7, a[illegible]opto[illegible]an be inhibited by autophagy inhibit[illegible] as 3-MA. However, autophagy may also inhibit apoptosis and decrease the [illegible]sis rate of tumor cells, and inhibition of [illegible]gy may improve the sensitivity of tumor cells to apoptotic signals. Moreover, autophagy and apoptosis collectively promote death of tumor cells, and once autophagy or apoptosis is inhibited, then switched to another cell death pathway[26,27]. Huang and Sinicrope[27] used celecoxib to treat human colorectal cancer cells and found that it could induce apoptosis and autophagy. In combination with Bcl-2/Bcl-xl antagonist, it was observed that it both apoptosis and autophagy were promoted, and the combination of celecoxib and autophagy inhibition could also promote apoptosis. In present study, combination of Met and IL-12 both promoted apoptosis and autophagy of tumor cells which may also be the one of the reasons for the synergistic effect.

Conclusions

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 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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