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Combination therapy with interleukin-15 and metformin as a synergistic treatment for pancreatic cancer

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Abstract. – OBJECTIVE: To investigate the efficacies and mechanisms of combination therapy with interleukin-15 (IL-15) and metformin (Met) on suppressing pancreatic cell proliferation and protecting Panc02-bearing mice.

MATERIALS AND METHODS: MTT assays were applied to assess the inhibitory effects of IL-15 combined Met or Met and IL-15 alone on proliferation of normal HPDE6-C7 and Panc02 cells. After tumor grew up to 150 mm^3, the Panc02-bearing xenograft model mice were randomly divided into saline group, IL-15 and Met alone group and combined treatment group (n=8) with the administration of each agent every other day for three weeks, and survival rates were recorded. The changes in tumor size, expression levels of the apoptosis, autophagy as well as Akt/mTOR/STAT3-related factors in tumor tissues were all measured.

RESULTS: MTT assays demonstrated significantly inhibiting efficacy of combination therapy with IL-15 and Met on Panc02 cell proliferation compared to other groups (all $p<0.05$) with combination index<1 showing evident synergistic effect. Moreover, the apoptosis rate of Panc02 cell under combined treatment were 95.5±3.2% and significantly higher than those of others (all $p<0.01$). In addition, combined administration remarkably inhibited the growth of pancreatic carcinoma, and improved survival rate of Panc02-bearing model with less body weight loss. Furthermore, combined treatment significantly downregulated anti-apoptotic proteins as well as Akt/mTOR/STAT3 signaling pathway and upregulated autophagy related factors, respectively, compared with those of monotherapy groups in both Panc02 cells and tumor tissues.

CONCLUSIONS: Combined treatment of IL-15 with Met showed synergistic anti-tumor efficacies on Panc02 cells attributing to promotion on apoptosis, autophagy and inhibition on Akt/mTOR/STAT3 signaling-transduction in Panc02-bearing model mice.

Key Words:
Interleukin-15, Metformin, Combined treatment, Panc02 cell, Apoptosis, Autophagy.

Introduction

Interleukin-15 (IL-15), as a multifunctional cytokine composed of 162 amino acids mainly exists in a high-affinity binding state with IL-15Ra, could promote the proliferation of activated T cells, regulate natural killing (NK) cells and induces the activity of cytotoxic T cells[1-3]. The anti-tumor effect of IL-15 is mainly achieved by promoting the proliferation and activation of CD8+ T cells and NK cells[4,5]. L15Rα presents IL-15 to IL-2/15Rβγ dimers to form a ternary complex, activates JAK and STAT signaling pathways, promotes elevated interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) secretion levels, and enhances the activation of CD8+ T cells and NK cells[6,7]. According to previous reports[8,9], IL-15 can promote tumor regression, reduce tumor metastasis and improve survival rate in a variety of experimental animal tumor models, including colon cancer and melanoma. Eason et al[10] demonstrated that IL-15 increased the number of CD8+ T cells in mice by promoting the proliferation of CD8+ T cells, while promoting the secretion of cytokines interferon (IFN) and tumor necrosis factor

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(TNF), ultimately enhancing the anti-tumor ability in hepatocellular carcinoma mice. Based on the good anti-tumor ability of IL-15, some authors[2] have begun to try to combine IL-15 or IL-15R agonists with other treatments in order to further improve the therapeutic effect of tumors, including combination with chemotherapeutic drugs or immune checkpoint blocking drugs.

Metformin (Met) is widely used as a first-line drug against diabetes all over the world with the advantages of low cost and low toxicity[11,12]. In recent years, many epidemiological and clinical studies[13-15] have shown that metformin can significantly reduce the risk of tumor and mortality. The anti-tumor mechanism of Met has not been fully clarified, which may be related to glucose metabolism and protein kinase pathways, but also to inflammatory factor-mediated tumor killing[16,17]. According to previous reports[18], the inhibitory effects of Met on tumor cell proliferation are mainly achieved through two aspects: (1) Indirect pathway: Met promotes mitosis by reducing circulating insulin levels, and insulin-like growth factor-1 pathway indirectly inhibits the proliferation of tumor cells. (2) Direct pathway: Met directly activates the adenosine-5-monophosphate-activated protein kinase (AMPK) pathway, inhibits the expression of its downstream protein mammalian target of rapamycin and P53 protein and then arrests the cell cycle to inhibit tumor cell proliferation[19].

Many patients with pancreatic cancer may have glucose metabolism disorders in the early stage[14,20]. According to clinical studies on the effect of metformin on the treatment of pancreatic cancer, some patients take metformin to control blood glucose while their risk of pancreatic cancer is also significantly reduced, as well as slowing down the progression of pancreatic cancer[21]. According to Liu et al[19], metformin can arrest pancreatic cancer cell cycle, inhibit pancreatic cancer cell proliferation, and affect pancreatic cancer cell invasiveness. Moreover, apoptosis of pancreatic cancer stem cells was evident, which may be related to activation of the AMPK pathway induced by Met[19]. In addition, metformin can induce cell cycle arrest in G0-G1 phase and inhibit cancer cell proliferation by downregulating the expression of cyclin D1, cyclin-dependent kinase 4, E2F1 transcription factors and upregulating the expression of p21[22].

As a potential anti-tumor drug, whether Met combined with IL-15 can play a synergistic anti-tumor effect is worthy of further exploration. However, no research on combined treatment of IL-15 and Met on pancreatic cancer has yet reported, let alone clarified the potent mechanism. Therefore, present study supplied novel approach for treating pancreatic cancer by investigating antitumor efficacies of IL-15 combined Met on proliferation, autophagy and apoptosis of Panc02 cell and tumor growth in Panc02-bearing mice.

Materials and Methods

Materials

Met powder were obtained from the Merck KGaA (Darmstadt, Germany). The IL-15 proteins with purity >98% were brought from R&D Company (Minneapolis, MN, USA). The *in vitro* reverse transcription kit, as well as PCR-related reagents were purchased from TaKaRa Bio Inc. (Kyoto, Japan). All PCR primers were synthesized by the GenWIZ (Suzhou, China). Rabbit anti-human caspase-3 and PCNA primary antibodies, rabbit anti-human B-cell lymphoma-2 (Bcl-2), BCL2-Associated X (Bax), survivin primary antibodies and mouse anti-human CyclinD1, β-actin primary antibodies as well as goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Abcam (Cambridge, UK). Other commonly used reagents were brought from Sigma-Aldrich (Darmstadt, Germany).

Animals

Fifty healthy BALB/C-nu/nu mice were purchased from Shanghai SLAC Laboratory Zoology Co., Ltd. (Shanghai, China), 4-5 weeks old, female, weighing 15-16 g (Certificate of Compliance No. 2018001580279). All mice were housed separately in a clean laminar flow rack in a barrier system with 4 mice per cage, with the environment controlled at room temperature 25±1°C and relative humidity of 40%-60%. The protocol and animal welfare of the animal experiments in the course of this trial were reviewed and approved by the ShengLunBio IACUC Committee with approval code of AC20192323CU. During the course of the trial, animal welfare and experimental procedures were performed in accordance with AAALAC's requirements.

Cell Strain and Cell Culture

The normal pancreatic ductal epithelial cells (HPDE6-C7, gift from Tsinghua University, Beijing, China) and mouse pancreatic cancer cell Panc02 (ATCC-MZ-0732) were stored in the liquid nitrogen. After cell recovery, the supernatant of Panc02 or HPDE6-C7 cells cultured in 25-mL

culture flasks was sucked clean, rinsed with phosphate-buffered saline (PBS), added with 0.25% trypsin solution and placed in a CO_2 incubator for about. 5 minutes later, remove the cells, gently shake to the cells and blow to suspension with a pipette, then add Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium (containing 10% fetal bovine serum) to terminate digestion, then suck the cells into a 15 mL centrifuge tube, centrifuge at 1200 rpm for 5 minutes, and then, discard the supernatant. 1-2 mL medium was added and cells were blown well, dispensed in 25 mL culture flasks and incubated in a CO_2 incubator. When the cell monolayer adhered and covered the bottom of the flask, it was digested and passaged again with trypsin until the number of cells required for the experiment was reached.

Cell Proliferation Inhibition Test

Metformin and IL-15 were added to HPDE6-C7 cells at final concentrations of 0, 0.3, 1, 3, 9, 27, 81 and 243 μg/mL and the final concentrations of 0, 01, 03, 0.1, 0.3, 0.9, 2.7 and 8.1 μg/mL, respectively, for 48 h of treatment. The effect of the combination on Panc02 cells was also investigated and grouped as follows: Negative control group, Met group (9, 27, 81 μg/mL), IL-15 group (0.3, 0.9, 2.7 μg/mL), combined treatment (27, 81 and 243 μg/mL), 3 wells for each sample. After treatment for 48 h, the reaction solution in the wells was sucked off, washed twice with sterile PBS, then 20 μL of 5 mg/mL MTT solution and corresponding culture medium were added to each well to 100 μL, and the culture was continued in an incubator at 37°C. After 4 h, the MTT culture solution was discarded, then 150 μL Dimethyl Sulfoxide (DMSO) was added to each well, shaken on a shaker at room temperature for 5 min, and the absorbance value of each well was detected with a microplate reader at a wavelength of 490 nm. According to the following formula, calculate the inhibition rate (IR, %) = (1-OD value of experimental group/OD value of control group) 100%. The two-drug interaction was calculated according to the formula: CI=sum of the inhibition rate of the combination of the two drugs/inhibition rate of the two drugs alone – product of the inhibition rate of the two drugs alone. CI < 0.85, antagonism of two drugs; $0.85 \leq CI \leq 1.15$, additive effect of two drugs; CI > 1.15, synergistic effect of two drugs.

Scratch Test

The Panc02 cells in logarithmic growth phase were trypsinized and the cell concentration was adjusted to about 5×10^5 cells/mL and seeded in 6-well plates and cultured in a 37°*C* constant temperature incubator. After 24 h of cell culture, monolayers were formed and spread to about 80% of the dish bottom, followed by a "one" scratch on the monolayer with a 100 μL pipette tip and washed three times with sterile PBS solution. The cells were randomly divided into negative control group, two single groups and combined treatment experimental group, 3 mL reaction system per well, with 3 parallel groups. The Panc02 cells were observed and randomly photographed under an inverted fluorescence microscope at 0 and 12 h. The scratch area was analyzed using Image J software to calculate the wound healing index = wound area (0 h)-wound area (12 h)/wound area (0 h).

Establishment of HepG2 Subcutaneous Xenograft Model

The female BALB/C mice aged 4-6 weeks, weighing 15-20 g, were adaptively reared. The Panc02 cells in the logarithmic growth phase were washed twice with PBS, and subsequently digested by adding 0.25% trypsin solution followed by placement in a CO_2 incubator. After 10 min, digestion was terminated with RPMI-1640 culture medium (containing 10% fetal bovine serum). The cell solution was centrifuged at 1200 rpm for 5 minutes, and the supernatant was discarded. Then, PBS was added again, the mixture was blown well and centrifuged for another 5 min, and the supernatant was discarded again. Serum-free RPMI 1640 culture medium was added to blow and beat the cells evenly, and they were prepared into single cells at a concentration of 2×10^7/mL in the case of cell viability > 95%. The preparation of mouse pancreatic cancer model was completed in a super clean bench, and aseptic operation should be paid attention to in the preparation process. Firstly, the skin of the right armpit of nude mice was disinfected with iodine tincture, and then 0.1 mL (about 2×10^6 cells) of cells was injected subcutaneously into the right armpit. After the completion of injection, the tumor formation was continuously observed, the body weight was measured every 3 days, and the long and short diameters of tumor tissues were measured each time with a vernier caliper. The transplanted tumor volume V (cm3) was calculated as: $V = 4\pi/3$ (tumor long diameter/2) × (tumor short diameter/2)^2. After ~1-2 weeks, the tumors with the volumes over 100 mm^3 were considered as successfully established.

Forty model mice were divided into negative control group, Met group (0.5 mg per mouse), IL-

15 group (5 μg per mouse), combination group (2.5 μg IL-15 and 0.25 mg Met per mice). The body weight changes were recorded twice a week. The size of tumor nodules was measured according above method. After 28 days of continuous medication, the tumor tissue samples were rapidly dissected and dissected from tumor-bearing mice, and the tumor inhibition rate was calculated after weighing the tumor weight in each group. Tumor inhibition rate (%) = [(average tumor weight of control group – average tumor weight of experimental group)/average tumor weight of control group] × 100%. The transplanted tumor specimens in each group were fixed in 10% formalin, embedded in paraffin, and serially pathologically sectioned for immunohistochemical detection. Some fresh tumor tissue was taken and stored in a liquid nitrogen tank for RT-PCR and Western blot detection.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

An appropriate amount of tumor tissue was ground evenly with 1 mL TRIzol, transferred into a 1.5 mL EP tube, and placed at room temperature for 5 min. Then, add 200 μL/mL chloroform, upside down, mix well and place it at room temperature for 15 min. Centrifuge the tissue fluid at 12000 g, 4°C for 15 min, then transfer the upper aqueous phase to another centrifuge tube, add an equal volume of isopropanol, mix well and place it at room temperature for 10 minutes. At 12,000 g, 4°C, the supernatant was discarded after centrifugation for 10 min, and 500 μL of absolute ethanol was added, centrifuged again and dried for 15 min. Subsequently, add 30 μL DEPC water for dissolution, and use a spectrophotometer to detect the absorbance value of organic substances such as nucleic acid and protein in the solution as well as the concentration of RNA.

The abundance of mRNA was assessed by conversion into complementary DNA and subsequent quantitative real-time PCR measurements using SYBR green-based fluorescence (TaKaRa Bio Inc., Japan). Relative quantification to housekeeping gene β-actin (ACTINB) was assessed with 2-DDCT method. The steps of mRNA expression amplification experiments were based on previous publications using the following primers: CyclinD1 (560bp): F upstream 5’-TGTTCGTGGCCTCTAAGATG-3’, R downstream 5’-ACTCCAGAAGGGCTTCAATC-3’; Bcl-2 (340bp): F upstream 5’-CGGGCATTCAGTGACCTGAC-3’, R downstream 5’-TCAGGAACCAGCGGTTGAAG-3’; Bax (312bp): F upstream 5’-GCGTCCAAGAAGCTGA-3’, R downstream 5’-ACCACCCTGGTCTTGGATCC-3’; Survivin (217bp): F upstream 5’-TACGCCTGTAATACCAGCAC-3’, R downstream 5’-TCTCCGCAGTTTCCTCAA-3’; Caspase-3 (309bp): F upstream 5’-GTCTCAATGCCACAGTCCAGT-3’, R downstream 5’-AGCAAACCTCAGGGAAACATT-3’; β-actin (308bp): F upstream 5’-AGCGGGAAATCGTGCGTG-3’, R downstream 5’-CAGGGTACATGGTGGTGCC-3’. Reaction conditions were as follows: 94°C for 10 min; 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s; 72°C for 10 min, 4°C Hold on. (The annealing temperature of β-actin, Bax, Bcl-2 and survivin was: 59°C; the annealing temperature of cyclin D1 and caspase-3 was: 60°C).

Western Blot Analysis

About 100 mg of fresh tumor tissues from each group were taken, added with 1000 μL of cell lysis solution (containing 50 × 20 μL of protease inhibitor) and placed on ice, homogenized every 5 minutes to fully lyse the tissues, for a total of 30 minutes of homogenization. Subsequently, we centrifuged at 14,000 rpm for 10 min at 4°C, collected the supernatant, determined the total protein concentration, dispensed into centrifuge tubes, and placed them at -20°C for cryopreservation. Cell supernatant were also stored at -20°C after determination of total protein concentration. The detailed procedures of Western blot analysis of tissues lysates or cell supernatant were performed according to previously reported method.

Statistical Analysis

SPSS 18.0 Statistical software (SPSS Inc., Armonk, NY, USA) was used for data analysis, and data were expressed as Mean ± SD. One-way ANOVA was used to compare two groups and multiple groups, and two-way ANOVA was used to compare random group data. $p<0.05$ indicated statistically significant difference.

Results

Combination of IL-15 and Met Exhibit Synergistic Inhibiting Effect on Panc02 Cell Proliferation

Firstly, we assess the cytotoxicity of Met and IL-15 on HPDE6-C7 cell, a normal human normal pancreatic ductal epithelial cell, *via* the MTT assay. As the results showed in Figure 1, con-

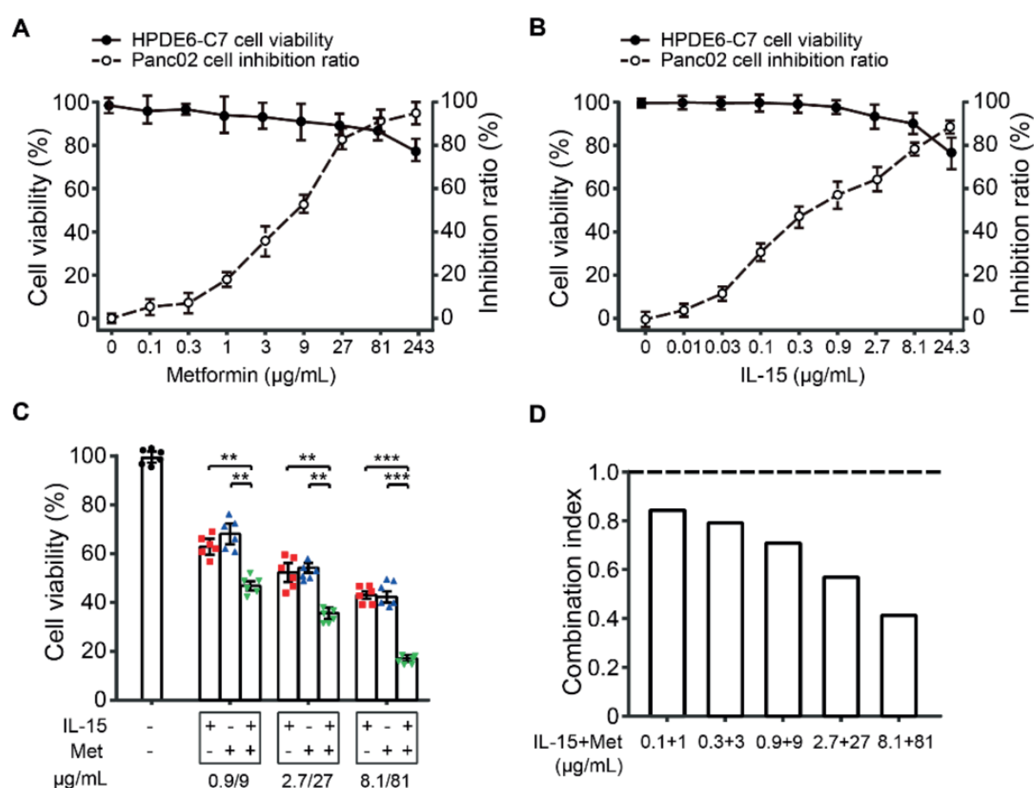

Figure 1. The protective effects of (**A**) MET alone, (**B**) IL-15 alone and (**C**) Met in combination with IL-15 on HPDE6-C7 cell viability and Panc02 cell apoptosis as well as (**D**) the calculated CI values. *$p<0.05$, **$p<0.01$, ***$p<0.001$ using one-way ANOVA. All data were presented as mean ± SD (n=6).

tinuously increased final concentration of IL-15 or Met did not induce significant effects on the cell viabilities within the range of 0 to 8.1 μg/mL and 0 to 81 μg/mL, respectively. In addition, the MTT assays performed in Panc02 cell demonstrated that the IL-15 and Met at different final concentrations exhibit the inhibiting efficacies on Panc02 cell proliferation. Not only that, the inhibition ratio of tumor cell proliferation was increased with gradually elevated final concentration of both two molecules with IC_{50} values of Met for ~8.3±1.5 μg/mL and IL-15 for ~0.5±0.1 μg/mL. Moreover, combined treatment of the IL-15 and Met showed obviously suppressed Panc02 cell viability compare to that of the monomer incubated cells (both $p<0.01$). Furthermore, we performed the calculation of the value of CI of the inhibiting efficacies of IL-15 combined Met on the proliferation Panc02 cell which was assessed by using the MTT method. As the data showed in the Figure 1, CI values of IL-15 and Met at all five combined doses are all less than 1.0, demonstrating that IL-15 holds synergistic effects with Met at above-mentioned doses on suppressing Panc02 cell proliferation.

Combination of IL-15 and Met Inhibit the Migration of Panc02 Cells

We further assessed the migration ability of Panc02 cells after treatment of Met and IL-15 alone or combination *via* cell scratch assay. As shown in Figure 2, the 12-hour scratch healing as well as its index of the Panc02 cells under the treatment of Met and IL-15 were both inhibited and the difference was statistically significant compared with that of the negative control group ($p<0.01$). Moreover, the combination of IL-15 and Met exhibited significantly enhanced inhibition on the scratch healing and its index of both two monomer treatment groups (both $p<0.05$) indicating the combination treatment synergistically suppressed the migration of Panc02 cells.

According previously reports, the Vimentin was positively correlated with tumor cell proliferation and metastasis, while e-cadherin was negatively correlated. Further western blot analysis

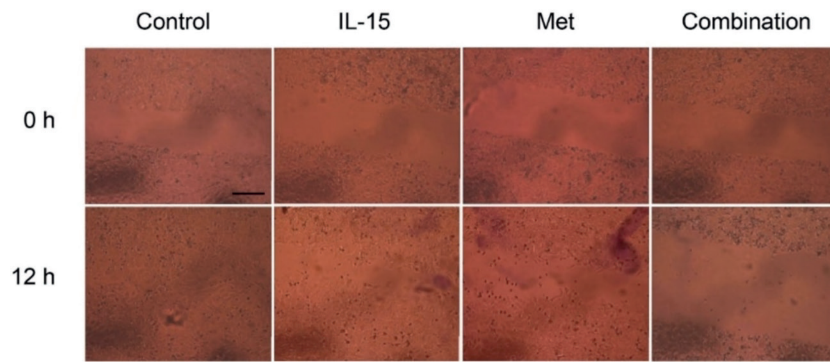

Figure 2. Effects of Combination of IL-15 and Met on inhibiting Panc02 cell migration. Scale bar, 20 µm.

was performed to assess the expression of the two biomarkers from Met or IL-15 alone treated Panc02 cells at 48 h. The expression levels of Vimentin and E-cadherin proteins were increased and decreased compared with the negative control group, respectively, and the differences were both statistically significant (Figure 3, both $p<0.01$). This experiment also showed that the expression of E-cadherin and vimentin in the cell supernatant was negatively correlated, suggesting the presence of EMT phenomenon and the inhibitory effects of IL-15 combined with Met on the proliferation and migration of Panc02 cells were stronger than those of the two monotherapy groups.

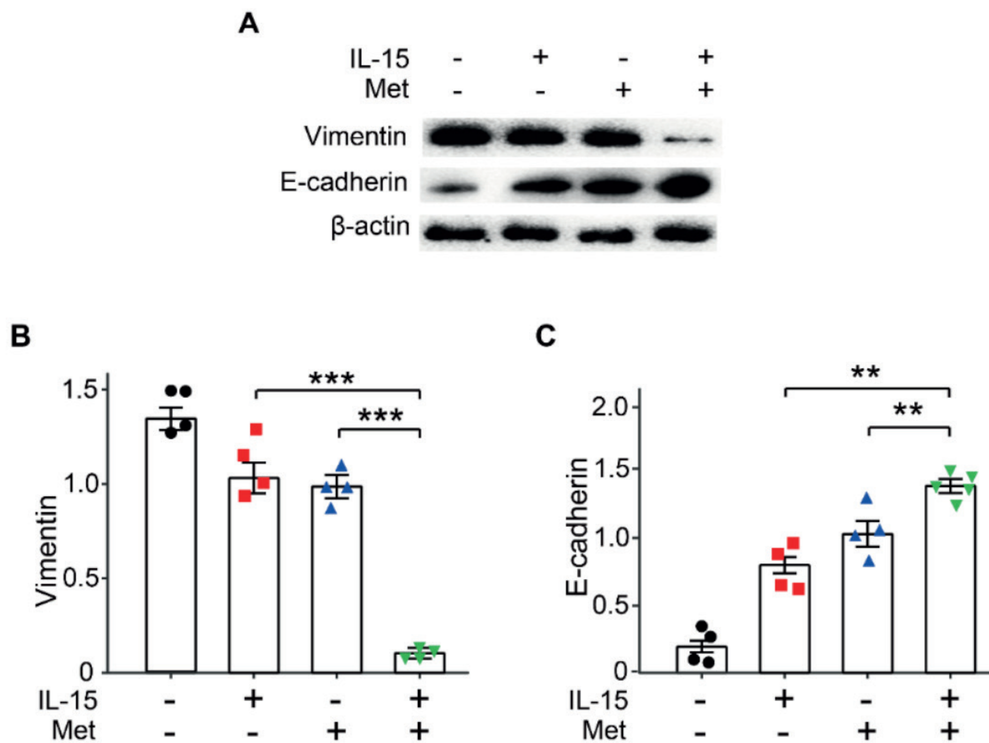

Figure 3. Effects of IL-15 in combination with Met treatment on expression levels of Vimentin and E-cadherin proteins. (**A**) Western blot analysis of (**B**) Vimentin and (**C**) E-cadherin. $^{**}p<0.01$ using one-way ANOVA. Results were showed as means ± SD (n = 4 each group).

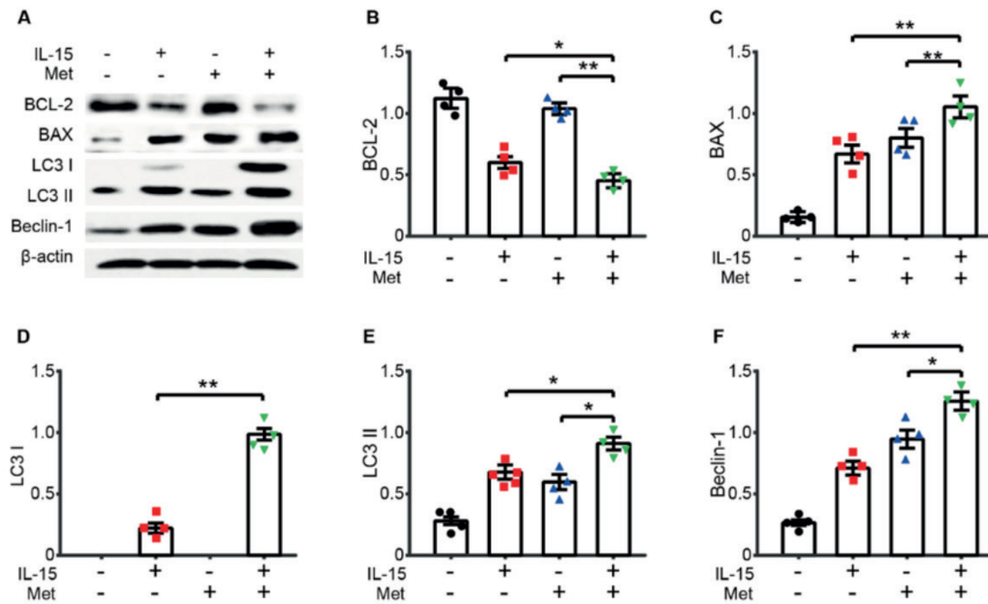

Figure 4. Western blot analysis of effects of combination of IL-15 and Met on apoptosis- and autophagy-related proteins in Panc02 cells. (**A**) Western blot analysis of (**B**) BCL-2, (**C**) BAX, (**D**) LC3 I, (**E**) LC3 II and (**F**) Beclin-1. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ using one-way ANOVA. Results were showed as means ± SD (n=4 each group).

Combination of IL-15 and Met Upregulate Expression Levels of Apoptosis-Related and Autophagy-Related Proteins in Panc02 Cell

Further investigation on the effects of IL-15 combined Met treatment on the expression levels of apoptosis-and autophagy-related biomarkers in drugs treated Panc02 cells were performed *via* Western blot method. As the data showed in Figure 4, monomer treatment of IL-15 or Met both downregulated the expression level of Bcl-2, as an anti-apoptotic protein, and upregulated expression level of Bax, as a pro-apoptotic protein. Moreover, combination incubation of IL-15 and Met exhibited significantly enhanced regulatory effects on the increase and decrease of the expression of Bcl-2 and Bax, respectively. Similarly, we detected the expression of autophagy marker protein, LC3, as well as autophagy phase related protein, Beclin1, in Panc02 cells. As the results showed in Figure 4, the expression level of Beclin1 and LC3 II were both remarkably upregulated in the pancreatic carcinoma Panc02 cells treated with IL-15 combined with Met compare to those of monomer treatment or negative control group. The results above demonstrate that combined incubation of Met and IL-15 can effectively promote the autophagy as well as apoptosis to finally inhibit the proliferation of pancreatic carcinoma Panc02 cells.

Combination of IL-15 and Met Effectively Inhibit the Growth of Pancreatic Cancer Xenografts

Consideration of the promising inhibitory efficacies of IL-15 combined with Met on the proliferation of Panc02 cell *in vitro*, further evaluation of this combination treatment to protect the mice from pancreatic carcinoma was performed in Panc02-bearing mice. Firstly, the pancreatic carcinoma model was successfully established after tumors gradually formed and exceeded 100 mm^3. Next, groups of Panc02-bearing mice were administrated with IL-15, Met or combination, and sera as well as tumor tissues were collected 50 days after initial treatment and subjected to further Western blot, H&E staining and immunohistochemical assays (Figure 5A). As was showed in Figure 5B, growth rate of the sizes of pancreatic carcinoma in mice were obviously suppressed by the treatment of IL-15 combined with Met and of which the inhibiting ratio was over 90% compare to those of saline-treated model mice ($p<0.01$). Remarkably, the tumor inhibiting ratio of the mice under the combined treatment (95.5%) was signifi-

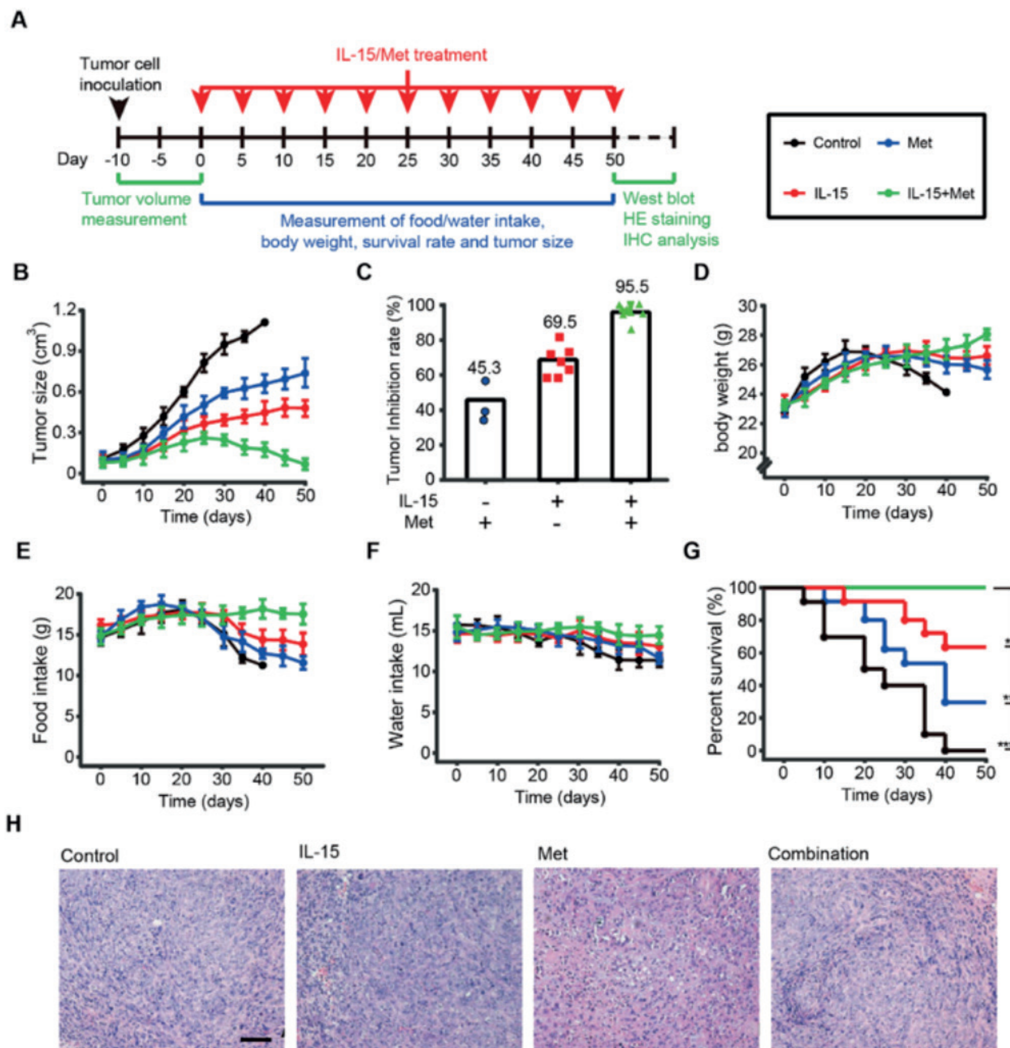

Figure 5. Chronic treatment of Met combined with IL-15 effectively inhibited pancreatic carcinoma growth in Panc02-bearing mice. (**A**) Schedule of animal experiment; (**B**) Tumor size-time curves in Panc02-bearing model mice; (**C**) tumor inhibition rate; (**D**) body weight and (**E**) food intake as well as (**F**) water intake of the Panc02-bearing model mice. (**G**) Survival curve and (**H**) HE analysis of pancreatic carcinoma tissues of Panc02-bearing mice. $p<0.05$, 0.01, 0.001 using one-way ANOVA (*, **, ***). Scale bar, 100 mm. All data are expressed as mean ± SD (n=10, 10 mice in each group).

cantly enhanced compared with that of model mice treated with IL-15 for 69.5% ($p<0.05$) or Met for 45.3% ($p<0.01$), respectively. As the results showed in the Figures 5C-D, body weights and food intakes of all Panc02-bearing model mice continuously increased in first two weeks, while the ones treated with saline gradually decreased with the increased volumes of tumors in the next five weeks. There was no significant difference in water intake among the groups of mice (Figure 5E). Moreover, the tumor-bearing mice under the 7-week combined or monomer treatment of Met and IL-15 exhibited distinct survival rates as 100% for combination group, 66.7% for IL-15 group, 33.3% for Met group, and 0% for negative control group (Figure 5G).

Following H&E staining, as well as IHC analysis of the tumor tissues, were performed. As shown in Figure 5H, the tumor cells in the negative control group were very abundant with inconsistent in size and shape, dark nuclei and large nuclear cytoplasmic ratio in nodular or woven arrangement. There was no evident pathological change, such as hemorrhage and necrosis were observed. In contrast, the pancreatic cancer cells in combination group were observed with significant signs of necrosis, which was significantly pronounced than those

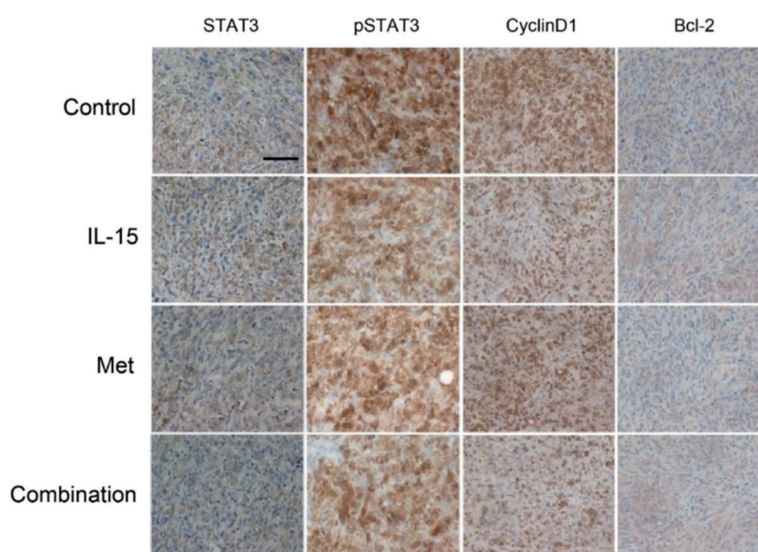

Figure 6. IHC analysis of the expression of Pstat3, Cyclin D1, Bcl-2 and STAT3 in the pancreatic carcinoma tissues from Panc02-bearing mice after 7-week treatment of Met combined with IL-15. Scale bar, 100 mm.

in the monotherapy group. We further used immunohistochemistry to detect STAT3, pSTAT3, Cyclin D1 and Bcl-2 protein expression levels in the xenograft tissues of each group (Figure 6). STAT3 phosphorylation is widely involved in tumorigenesis in a variety of tissues and aberrantly activated in a variety of malignancies. Previous reports demonstrated that in malignant tumor cells, STAT3 can induce cell cycle and proliferation gene Cyclin D1 as well as proto-oncogene C-myc expression to promote cell proliferation, and can upregulate the anti-apoptotic gene Bcl-2 to inhibit apoptosis of tumor cells. Each histone immunoreactive material showed brown to tan coloration. In the tumor tissues after the intervention of IL-15 combined Met, the expression of STAT3 was not significantly different from that in the negative control group and the two groups (all $p>0.05$), while the expression of Bcl-2 and pSTAT3 were significantly lower than those in the negative control group and the two groups (all $p<0.05$). Moreover, the expression of Cyclin D1 was only significantly decreased compared with the negative control group ($p<0.05$), and there was no significant difference compared with the single group ($p>0.05$).

Combination of IL-15 and Met Enhanced Apoptosis of Panc02 Tumor Cell in Mice

We further investigated the expression of Bax, Bcl-2, Survivin, CyclinD1 and Caspase-3 genes in the tumor tissues from all four groups. As the results showed in Figure 7, the expression of Bcl-2, Survivin and CyclinD1 genes were significantly downregulated, while the expression of Bax and caspase-3 genes were obviously upregulated in the combination treatment group compared with the control group ($p<0.01$) and monomer treatment groups (both $p<0.05$). Similarly, the Western blot results showed in Figure 8 also demonstrated the upregulation of protein levels of Bax and Caspase-3, and downregulation of the protein levels of Bcl-2, Survivin and CyclinD1, which were all consistent with the results of each mRNA, suggesting that IL-15 synergizes with Met to affect the expression of the above genes and then jointly promote apoptosis, inhibit cell proliferation, and finally inhibit the growth of subcutaneous xenografts of pancreatic cancer cell line Panc02.

Combination of IL-15 and Met enhanced autophagy and inhibited AKT/mTOR/STAT3 signaling pathway in Panc02 tumor

Zhao et al[23] demonstrated that Met can promote autophagy through AMPK/mTORc1 signaling pathway to inhibit the cancer cell proliferation. Therefore, we investigated the effects of combination treatment of IL-15 and Met on autophagy of tumor cells by detecting the marker protein including LC3 and Beclin-1 in pancreatic carcinoma tissues from all four

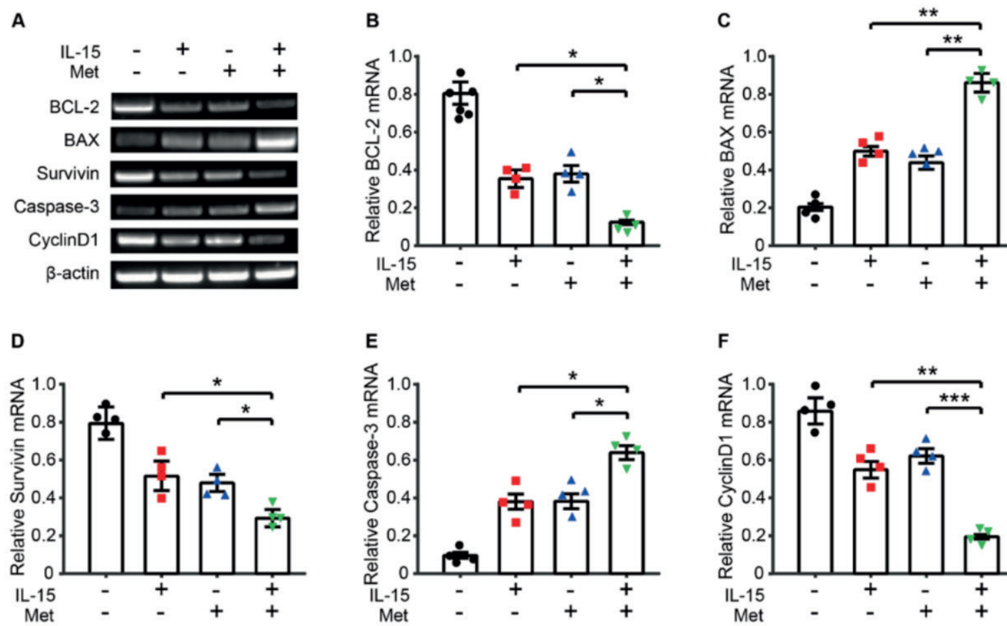

Figure 7. Effects of Met, IL-15 and combination on the expression of Bcl-2, Bax, Survivin, Caspase-3 and CyclinD1 mRNA in tumor-bearing mice. The (**A**) agarose gel electrophoresis analysis of (**B**) Bcl-2; (**C**) BAX; (**D**) Survivin; (**E**) Caspase-3 and (**F**) CyclinD1. $p<0.05$, 0.01, 0.001 using one-way ANOVA (*, **, ***). All data are expressed as mean ± SD.

groups. As the results showed in Figure 9, the expression levels of LC3 II, as well as Beclin-1 proteins were both significantly upregulated in combined treatment group compare to those of the negative control and monotherapy groups.

Lin et al[24] previously reported that both IL-15 and Met can induce autophagy in tumor cells by inhibiting AKT/mTOR/STAT3 signaling pathway, which in turn inhibits the proliferation of Hepatic carcinoma cells. Combining the above

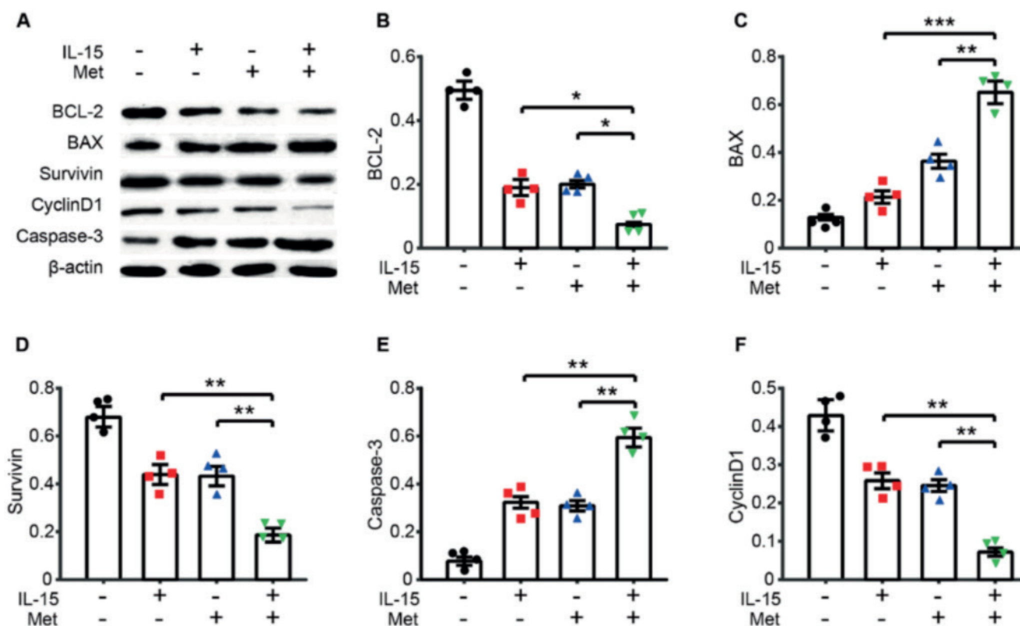

Figure 8. Effects of Met, IL-15 and combination on the expression of Bcl-2, Bax, Survivin, Caspase-3 and CyclinD1 protein in tumor-bearing mice. The (**A**) Western blot analysis of (**B**) Bcl-2; (**C**) BAX; (**D**) Survivin; (**E**) Caspase-3 and (**F**) CyclinD1 protein. $p<0.05$, 0.01, 0.001 using one-way ANOVA (*, **, ***). All data are expressed as mean ± SD.

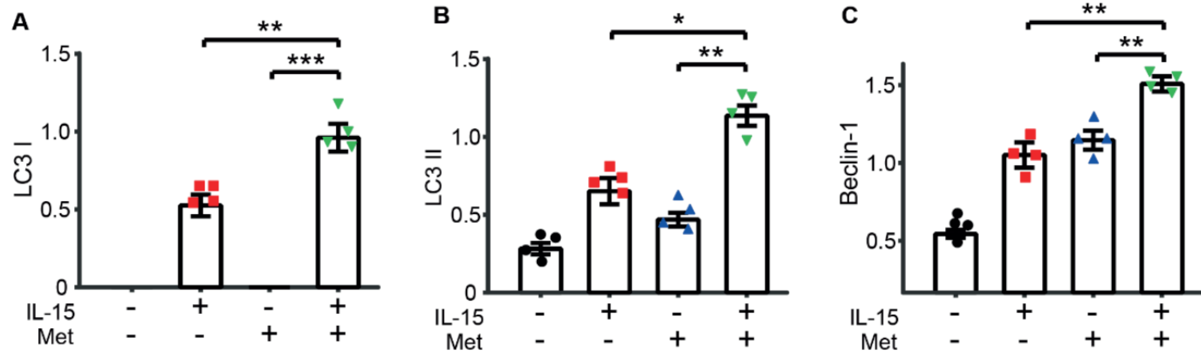

Figure 9. Effects of Met, IL-15 and combination on autophagy-related proteins in tumor tissues. The western blot analysis of (**A**) LC3 I; (**B**) LC3 II and (**C**) Beclin-1. $p<0.01$, 0.001 using one-way ANOVA (*, **, ***). All data are expressed as mean ± SD.

results of which the combination treatment can significantly increase the autophagy level of Panc02 cells, we therefore need to determine whether the combination of IL-15 and Met can also effectively affect the AKT/mTOR/STAT3 signaling pathway by detecting the expression levels of related proteins in tumor tissues from all four groups. As shown in Figure 10, combination treatment significantly suppressed the expression levels of all phosphorylated AKT, mTOR and STAT3 proteins indicating that the AKT/mTOR/STAT3 signaling pathway was indeed inhibited.

Discussion

Pancreatic cancer is a highly malignant malignancy of the digestive tract, about 90% of which are ductal adenocarcinomas arising from the

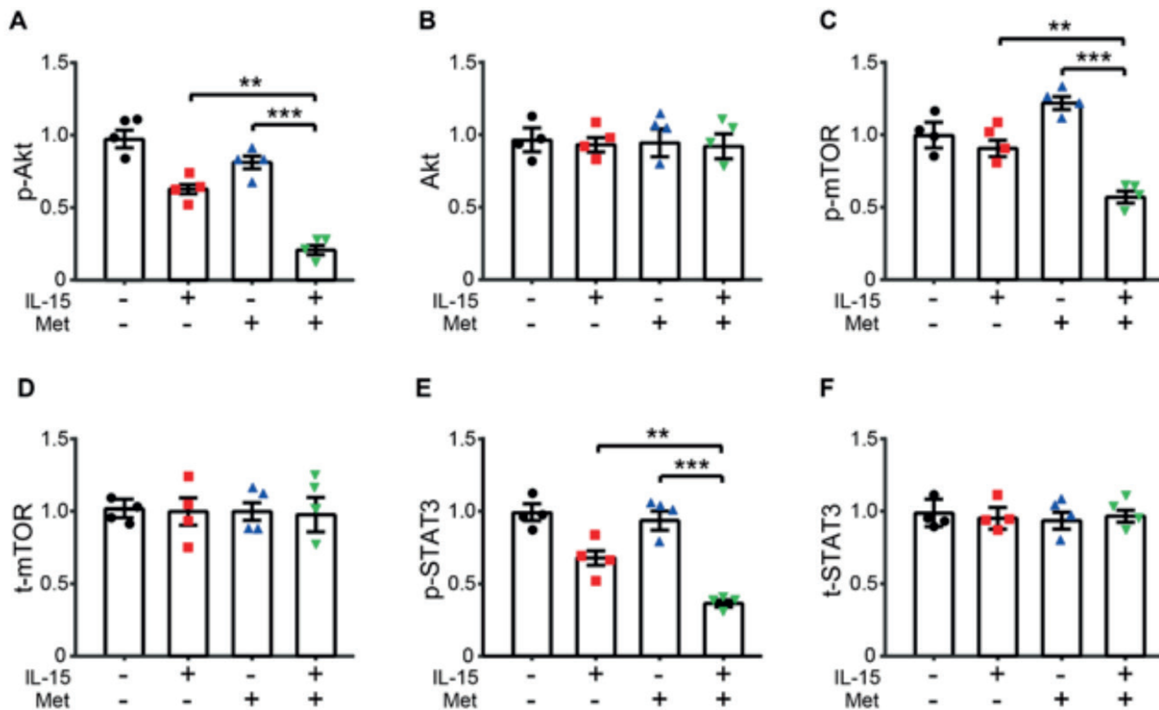

Figure 10. Effects of Met, IL-15 and combination on Akt/mTOR/STAT3 signaling pathway in tumor tissues. The Western blot analysis of (**A**) p-Akt; (**B**) Akt, (**C**) p-mTOR, (**D**) t-mTOR, (**E**) p-STAT3 and (**F**) t-STAT3. $p<0.01$, 0.001 using one-way ANOVA (**, ***). All data are expressed as mean ± SD.

glandular tubular epithelium, with a high mortality rate, a very poor prognosis, and a 5-year survival rate of less than 1%[25,26]. There are few effective treatments for pancreatic cancer and the prognosis is poor, so it is important to find new therapeutic targets and treatments[27].

Diabetes mellitus is an important factor in the pathogenesis of pancreatic cancer, and the prognosis of patients with diabetes mellitus combined with pancreatic cancer is poor[28,29]. Therefore, the search for new targets for pancreatic cancer treatment in diabetes-related researches is a current research hotspot. Metformin, as a most commonly used antidiabetic agent, was previously reported with certain therapeutic effects on colorectal cancer, breast cancer, ovarian cancer, endometrial cancer as well as pancreatic cancer[16,17]. The mechanism by which Met improves the pathogenesis and prognosis of pancreatic cancer is complex and there is no uniform theory[17]. Current researches[17,30,31] of the mechanism of Met on tumor focus on insulin-like growth factor, AMP-activated kinase (AMPK) pathway, and rapamycin target (mTOR) pathway. Biadgo et al[32] have suggested that the increased incidence and worse prognosis of pancreatic cancer in diabetic patients may be related to the hyperglycemic state and hyperinsulinemia. Met could inhibit the proliferation of pancreatic cancer cells by decreasing insulin levels, leading to inactivation of insulin-like receptor substrate-1, inhibition of insulin-like growth factor-1 (IGF-1), and improvement of glucose and insulin metabolisms[18]. Under physiological conditions, adenosine triphosphate (ATP) at low level can activate AMPK and positively regulate gluconeogenesis, fatty acid oxidation and autophagy[33]. In addition, the activation of AMPK can also lead to the activation of p53, which promotes apoptosis and autophagy[33]. Met can activate inactivated AMPK in pancreatic cancer, thereby inhibiting the release of pro-fibrogenic factors by pancreatic cancer cells, as well as the paracrine effect of pancreatic stellate cells, inhibiting the formation of connective tissue, and inhibiting the growth of tumors, thereby improving the prognosis of pancreatic cancer[16,34].

In present study, MTT assays demonstrated that continuously increased concentration of IL-15 or Met did not affected the cell viabilities of HPDE6-C7, a normal human normal pancreatic ductal epithelial cell, until 8.1 and 81 μg/mL, respectively. In addition, incubation of IL-15 and Met at different final concentrations significantly inhibits the proliferation of Panc02 cells with IC_{50} values of Met for ~8.3±1.5 μg/mL and IL-15 for 0.5±0.1 μg/mL. Remarkably, combined incubation exhibited significant effects on the inhibition rate of the cell viabilities of Panc02 cell line which was statistically higher than that of tumor cells treated with (both $p<0.01$). Similarly, the calculated CI values of inhibitory efficacies of combined IL-15 with Met on *in vitro* cell proliferation of Panc02 were all less than 1.0, suggesting the synergistic inhibition effects of combination incubation.

The tumor cells hold the ability of continuous proliferation and metastasis, and the Panc02 cell line also has a certain ability of proliferation and migration. To understand whether the combination of IL-15 and Met exhibited an effect on the migration of pancreatic cancer cells, we further performed a scratch assay. Further results exhibited that Panc02 cells had significantly migrated after 12 h of blank control scratch, while incubation of IL-15 and Met alone could inhibit this migration. At the same time, the healing area ratio and cell healing area ratio (cell healing index) of IL-15 and Met alone was smaller than that of negative control with the statistically significant difference ($p<0.05$). Moreover, the combination effect was that the two drugs played a synergistic role, which indicated that Met could enhance the inhibitory effect of IL-15 on the migration of pancreatic adenocarcinoma Panc02 cells. Invasion and metastasis are important features of tumors leading to disease deterioration and accelerate patient death. Tumor invasion and metastasis is a very complex continuous multi-step process. Numerous studies have reported that epithelial-mesenchymal transition (EMT) is extremely closely related to tumor invasion and metastasis, and is one of its important mechanisms. However, the link between Met, IL-15 and EMT in pancreatic cancer has not been reported. In this study, scratch assay has verified that Met and IL-15 can inhibit the migration of pancreatic cancer Panc02 cells, and the combined effect is more evident. To investigate its specific molecular mechanism, subsequent Western blot experiments were performed in this experiment. Semi-quantitative analysis showed that the expression of E-cadherin in Panc02 cells treated with Met and IL-15 was significantly higher than that in the negative control, while the expression of vimentin was significantly lower in Met cells (all $p<0.05$). The expression of E-cadherin was the highest and the expression of vimentin was the lowest in the Met combined with IL-15 group, and the difference was statistically significant

compared with the two single groups (all $p<0.01$), suggesting that the combination of Met and IL-15 reduced the migration ability of cells and was significantly better than the two single groups, and may be related to the reversal of EMT.

It has been demonstrated that combination of Met or IL-15 with radiotherapy or chemotherapy exhibited synergistic effects in various tumor cells which mainly via enhancing the apoptosis and inhibiting the proliferation of tumor cells. Apoptosis, as a cell-autonomous ordered death controlled by genes for maintaining homeostasis and most antitumor agents, including Met and IL-15 can effectively induce apoptosis accompanied by several changes, including cell membrane invagination, cytoplasmic condensation and apoptotic body formation. The regulatory mechanism of apoptosis is extremely complex, and its most common apoptosis-related proteins are Bcl-2 and Bax. The Bcl-2 protein localizes to mitochondria, endoplasmic reticulum, as well as nuclear membranes in different tumor cells and exerts anti-apoptotic effects by preventing the release of mitochondrial cytochrome. In addition, Bcl-2 has the function of protecting cells, and overexpression of Bcl-2 can cause the accumulation of nuclear reduced glutathione, resulting in changes in the redox balance in the nucleus, which reduces the activity of caspase. Bax is a member of the Bcl-2 family involved in apoptosis, and when apoptosis is induced, it migrates from the cytosol to mitochondria and nuclear membranes to form homodimers that promote apoptosis. Yang et al[35] previously reported that Met suppressed the tumor cell proliferation including HepG2 cell line by suppressing the expression level of Bcl-2 protein to realize the antitumor efficacy. At present, Western blot analysis demonstrated that combined Met with IL-15 significantly downregulated and upregulated the protein expression levels of Bcl-2 and Bax, respectively, compare to those of the negative control and two monotherapy groups (all $p<0.05$) showing enhanced apoptosis in Panc02 cells. Moreover, in tumor cells, autophagy can protect damaged tissues by inhibiting cancer cell proliferation and promoting their apoptosis, which in turn reduces tumor progression such as inflammatory response and tissue damage. Furthermore, the protein expression levels of LC3 and Beclin-1 were significantly upregulated in tumor tissues from combination treatment of Met with IL-15 which was also significantly higher than those in monomer-treated or saline-treated group.

In the *in vivo* pharmacodynamic evaluation, we found that the combination of IL-15 and Met was very effective in the treatment of pancreatic cancer in mice, including significantly inhibiting the growth rate of tumors, stabilizing the body weight and food intake of tumor-bearing mice and greatly improving the survival rate, and the above effects were more significant than those of the monotherapy group. In order to explain the above phenomenon, we further explored the mechanism by which the combination treatment of Met and IL-15 enhances the anti-pancreatic cancer effect.

According to previous studies, we found that STAT3 induced cell cycle and proliferation gene Cyclin D1 and proto-oncogene C-myc expression to promote cell proliferation, and upregulated the anti-apoptotic gene Bcl-2 and downregulated the pro-apoptotic gene Bax to inhibit apoptosis in malignant cells. Cyclin D1, as a proto-oncogene encoding cyclin, is not only involved in cell proliferation and growth regulation, but also regulates important links such as mitochondrial activity, DNA repair and cell migration. Under normal conditions, Cyclin D1 will be decomposed from the nucleus into the cytoplasm. If Cyclin D1 expression is increased after activation, it shortens G1 phase and promotes the cell cycle to enter S phase in advance, resulting in uncontrolled cell proliferation and leading to the occurrence of tumors. Survivin is a member of the inhibitor of apoptosis protein family (IAP), which is considered to be a nodal protein that is able to both inhibit apoptosis and regulate the mitosis of cells.

In this study, we found that the mRNA and protein expression levels of CyclinD1, Bcl-2 and Survivin in the tumor tissues from combination treatment groups were significantly downregulated, while the expression of Caspase-3 and CyclinD1 were significantly upregulated compared with those of the control group and monotherapy group. Immunohistochemical detection also showed that Bcl-2 protein expression was downregulated, while caspase-3 protein expression was upregulated in the single and combined groups. Thus, the mechanism of better antitumor effect of the combination may be related to the induction of tumor cell apoptosis and the inhibition of tumor cell proliferation. Moreover, the results of immunohistochemistry and Western blot showed that the expression of STAT3 in tumor tissues of each group had no significant change, while the expression of pSTAT3 in tumor tissues was significantly decreased after combined administration of Met and IL-15, which was significantly lower than those of the control group and two monotherapy

group. Similar to previous findings, STAT3 phosphorylation is hyperactivated in a variety of malignancies. We, therefore, reasoned that Met and IL-15 may promote pancreatic cancer xenograft growth by inhibiting STAT3-related signaling pathways by decreasing STAT3 phosphorylation (pSTAT3). Furthermore, we speculated that the combination may enhance autophagy and apoptosis of Panc02 cells by inhibiting AKT/mTOR/STAT3 signaling pathway, which in turn plays a role in inhibiting tumor cell growth and invasion. Interestingly, we did detect an inhibitory effect of the combination on AKT, mTOR and STAT3 protein phosphorylation by Western blot.

In summary, combination treatment of Met and IL-15 indeed exhibited synergistic antitumor effects on proliferation of Panco2 cells which were significantly enhanced compared with monomer treatment. The main mechanism may be attributed to the inhibition on cell invasion and Akt/mTOR/STAT3 signaling pathway which further induce the apoptosis and autophagy in Panc02 cells or tumor tissues. The greatest innovation of this study lies in our finding for the first time that the combination of Met and IL-15 has a synergistic effect on inhibiting pancreatic cancer and its mechanism has been intensively studied. Our finding will supply the pharmacodynamic and theoretical basis for future clinical usage of these two agents for treating pancreatic cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Author Contributions

Conceptualization, L. Shi and J. Wen; Data curation, L. Shi and J. Wen; Methodology, Q.-H. Xu, L.-J. Zhou and G.-H. Yang; Data analysis, Q.-H. Xu, L.-J. Zhou, G.-H. Yang, F.-D. Meng, Y. Wan, L. Wang; Supervision, Q.-H. Xu and F.-D. Meng; Validation, L. Zhang and Q.-H. Xu; Writing-original draft, Q.-H. Xu; Writing-review & editing, L. Shi and J. Wen.

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