

# TGF- $\beta$ 1 promotes human hepatic carcinoma HepG2 cells invasion by upregulating autophagy

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**Abstract. – OBJECTIVE:** To study the role of TGF- $\beta$ 1 in autophagy and invasion ability of human hepatic carcinoma HepG2 cells.

**MATERIALS AND METHODS:** Cultured HepG2 cells were treated with different concentrations of TGF- $\beta$ 1 for 24 h. The protein expression levels of autophagy relative marker LC3 and Beclin1 were detected by Western blot. The effect of TGF- $\beta$ 1 on invasion ability of HepG2 cells was detected with transwell method.

**RESULTS:** The results demonstrated that TGF- $\beta$ 1 was able to activate autophagy of HepG2 cells in a dose-dependent manner. Autophagy inhibitor 3-methyladenine (3-MA) could reverse TGF- $\beta$ 1 induced autophagy process. Also, TGF- $\beta$ 1 significantly promotes the invasion ability of HepG2 cells; however, this process could effectively reverse by autophagy inhibitor 3-MA.

**CONCLUSIONS:** TGF- $\beta$ 1 enhances HepG2 cells invasion by upregulating autophagy.

*Key Words:*

Human hepatic carcinoma, Autophagy, Transforming growth factor, Invasion.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in clinical practice. In recent years, liver cancer has become the carcinoma featured with incidence rate and mortality rate in China, and its mortality rate is just secondary to the lung cancer<sup>1</sup>. With the development of the medical concept and treatment method of tumor, some achievements have been made in the early diagnosis and treatment of liver cancer, but most of patients with liver cancer in middle and advanced stages still suffer from the insufficient and ineffective therapeutic methods. In the advanced stage of liver cancer, cell invasion easily occurs in the capsule and

vessels surrounding the tumors, leading to the local spreading and distant metastasis<sup>2</sup>. Thus, investigating the mechanisms of occurrence, development, invasion and metastasis of liver cancer is of great significance for improving the prognosis of patients. Autophagy refers to a kind of self-protection mechanism of cells in response to various stress states, such as nutrient insufficiency, infection, oxidative stress and insufficient energy metabolism. Through autophagy, injured organelles and mitochondria inside the cells can be degraded into the absorbable biomacromolecules that can be utilized to provide the nutrient support for cells, thus maintaining the survival of cells under stress state<sup>3</sup>. However, excessive activation of autophagy may cause injuries of cells and induce the autophagic death of cells<sup>4</sup>. A research<sup>5</sup> has shown that autophagy plays roles in all the tumor stages, such as formation, occurrence, development, invasion and metastasis of tumors. Transforming growth factor (TGF), a kind of polypeptide molecules with multiple biological effects that are secreted by a variety of cells, can exert the regulatory functions in cell proliferation and differentiation, and participate in key biological processes, such as angiogenesis, fibrosis in damage repair and occurrence of tumor<sup>6</sup>. TGF- $\beta$  can be divided into 4 subgroups: TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3 and TGF- $\beta$ 1 $\beta$ 2. Among these subgroups, the expression of TGF- $\beta$ 1 is the highest. The inhibitory effect on tumor of TGF- $\beta$  is exerted in the early stage of tumor occurrence, but it can promote the growth, invasion and distant metastasis of tumor after the formation of tumor lesion<sup>7</sup>. Also, the research<sup>8</sup> shows that TGF- $\beta$ 1 is involved in the regulation of autophagic activity. In this study, we aimed to investigate the regulatory effect of TGF- $\beta$ 1 of the autophagic activity of HpeG2 cell in HCC and the impact

on the invasion capability to provide the experimental basis for further studies on the regulation mechanism of TGF- $\beta$ 1 in the metastasis of liver cancer.

## Materials and Methods

### Material

HpeG2 cells of liver cancer (American Type Culture Collection Center, Manassas, VA, USA); TGF- $\beta$ 1 (R&D, Minneapolis, MN, USA); 3-Methyladenine (3-MA, Selleck Chemicals Co., Ltd., Houston, TX, USA); polyclonal rabbit anti-human antibody of microtubule-associated protein 1 light chain 3 (LC3); polyclonal rabbit anti-human Beclin1 antibody (Abcam, Cambridge, MA, USA); antibody of  $\beta$ -actin (Wuhan Sanying, Hubei, China); goat anti-rabbit secondary antibody (Beijing Zhongshan Goldenbridge Biotech Co., Ltd., Beijing, China); protein markers (Thermo Fisher Co., Ltd., Waltham, MA, USA); trypsin in 0.025% EDTA (Wuhan Boster Bioengineering Co., Ltd, Hubei, China); ECL kit (Millipore, Billerica, MA, USA); fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China); Dulbecco's Modified Eagle Medium (DMEM) (high-glucose) (Hyclone, Logan, UT, USA); RIPA kit and BCA protein assay kit (Beyotime Institute of Biotechnology, Beijing, China); 24-well transwell chamber (core diameter: 8  $\mu$ m; Corning Costar, Corning, NY, USA); matrigel for invasion experiment (BD, Boston, MA, USA).

### Cell Culture

In a cell incubator (37°C, 5% CO<sub>2</sub>, saturated humidity), HpeG2 cells of liver cancer were cultured in the Dulbecco's Modified Eagle Medium (DMEM) culture containing 10% deactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100  $\mu$ g/mL gentamicin. Then cells were digested and passaged using 0.025% trypsin. When 70 to 80% of cells were merged, those cells were starved overnight using the serum-free culture medium. Thereafter, TGF- $\beta$ 1 was added for intervention, and the cells were divided into groups according to the experiment requirement.

### Western Blot Detection

After being treated using TGF- $\beta$ 1 in different concentrations (0 ng/mL, 5 ng/mL, and 10 ng/mL) for 24 h, cells that adhered to the walls of culture bottle were washed using pre-heated

phosphate buffered saline (PBS) and transferred to the Eppendorf (EP) tubes for centrifugation at 12000 rpm for 5 min. Then, the supernatant was discarded, and cells were added into the radioimmunoprecipitation assay (RIPA) for cell lysis. The supernatant was taken for preservation at -20°C. Samples were loaded, and proteins were gathered via 80V electrophoresis. Under the voltage of 100V, proteins were isolated and transferred onto the membrane. The membrane was blocked using 5% skimmed milk for 1 h. After, the proteins on the membrane were incubated using polyclonal rabbit anti-human antibody LC3B (1:1000) and polyclonal rabbit anti-human Beclin1 antibody overnight at 4°C. Then, the membrane was washed in the shaking bed using tris buffered saline-tween (TBST) for decolorization for 3 times (5 min/time). Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG was added onto the membrane for incubation at room temperature for 1h followed by washing the membrane using TBST for 3 times (5 min/time). After electrochemiluminescence (ECL) was added onto the membrane for reaction of 1 min, the membrane was exposed under the X-ray followed by fixation and image development.

### Cell Invasion Experiment

Before experiment, matrigel was melted at 4°C. 40  $\mu$ L matrigel that were diluted using serum-free culture media (1:3) were spread on the surface of polycarbonate micropore. Then, the matrigel was placed in an incubator for 4 h of coagulation and preservation. Human liver cancer HpeG2 cells in the logarithm phase were starved in the serum-free Dulbecco's Modified Eagle Medium (DMEM) culture medium for 24 h, and digested using 0.025% EDTA trypsin. Single-cell suspension was prepared using the cells and serum-free DMEM culture medium. Cell density was adjusted to  $2 \times 10^5$  /mL, and the cell viability that was detected via trypan blue staining was above 95%. In the upper chamber of each invasion chamber, 200  $\mu$ L serum-free cell suspension was added. Meanwhile, drugs were added according to the experimental requirement of each group. 3 replicate pores were set in each group. In the lower chamber, DMEM culture medium containing 10% fetal bovine serum (FBS) was added (600  $\mu$ L in each hole). The plate was then transferred to the incubator for 24 h of cell incubation. The plate was taken out and washed using phosphate buffered saline (PBS) for 2 times. Then, the medium

was removed, and cells that did not penetrate the upper layer of chamber were wiped using wet cotton swabs. After 20 min of fixation using methanol, the plate was dried at room temperature and was stained using Coomassie brilliant blue (CBB) for 20 min. Thereafter, cell count was performed under the inverted microscope to figure out the quantity of cells that penetrate the membrane. Cells in the upper, lower, left, right and middle magnification fields (400 $\times$ ) were counted and the average was taken.

### Statistical Analysis

All values were presented as mean  $\pm$  standard deviation. Single-factor ANOVA was performed using SPSS 22.0 software (SPSS Inc. Chicago, IL, USA) in the statistical analysis.  $p < 0.05$  suggested that the difference had statistical significance. Tukey's HSD (honestly significant difference) test is used in conjunction with an ANOVA to find means that are significantly different from each other.

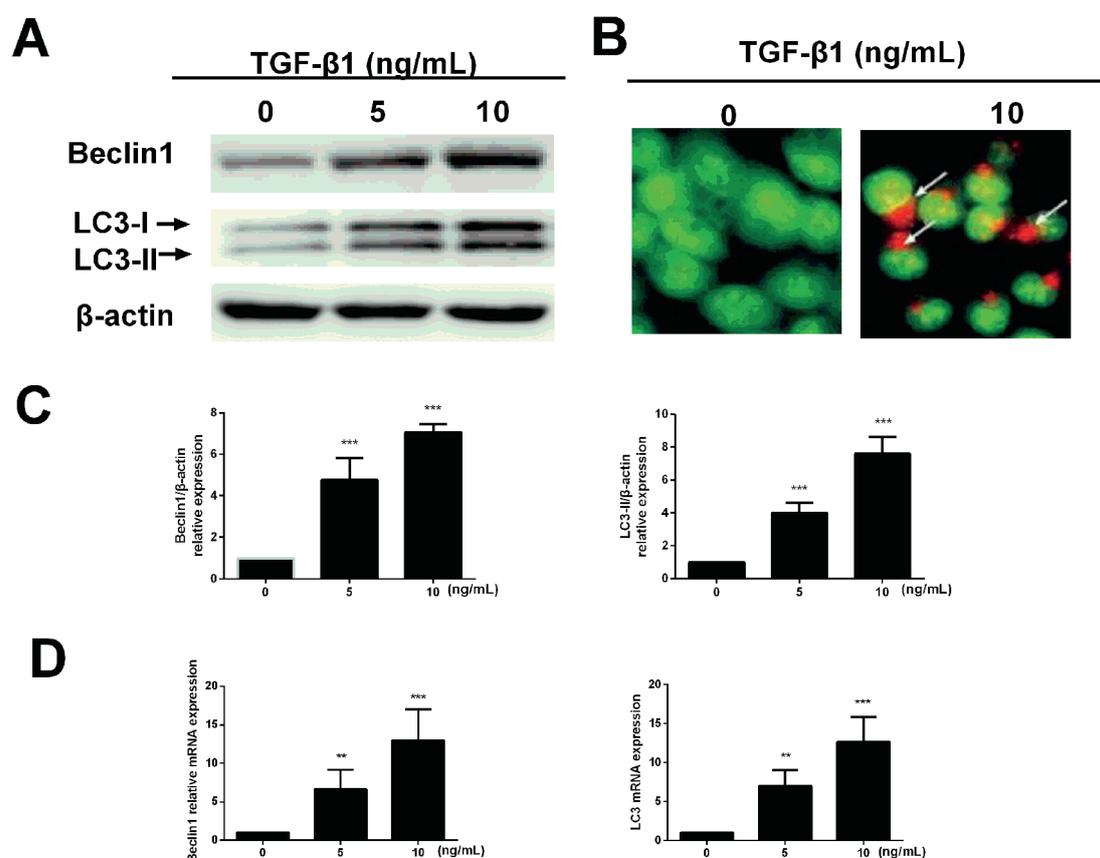
## Results

### TGF- $\beta$ 1 Inducing the HpeG2 Cell Autophagy of Liver Cancer

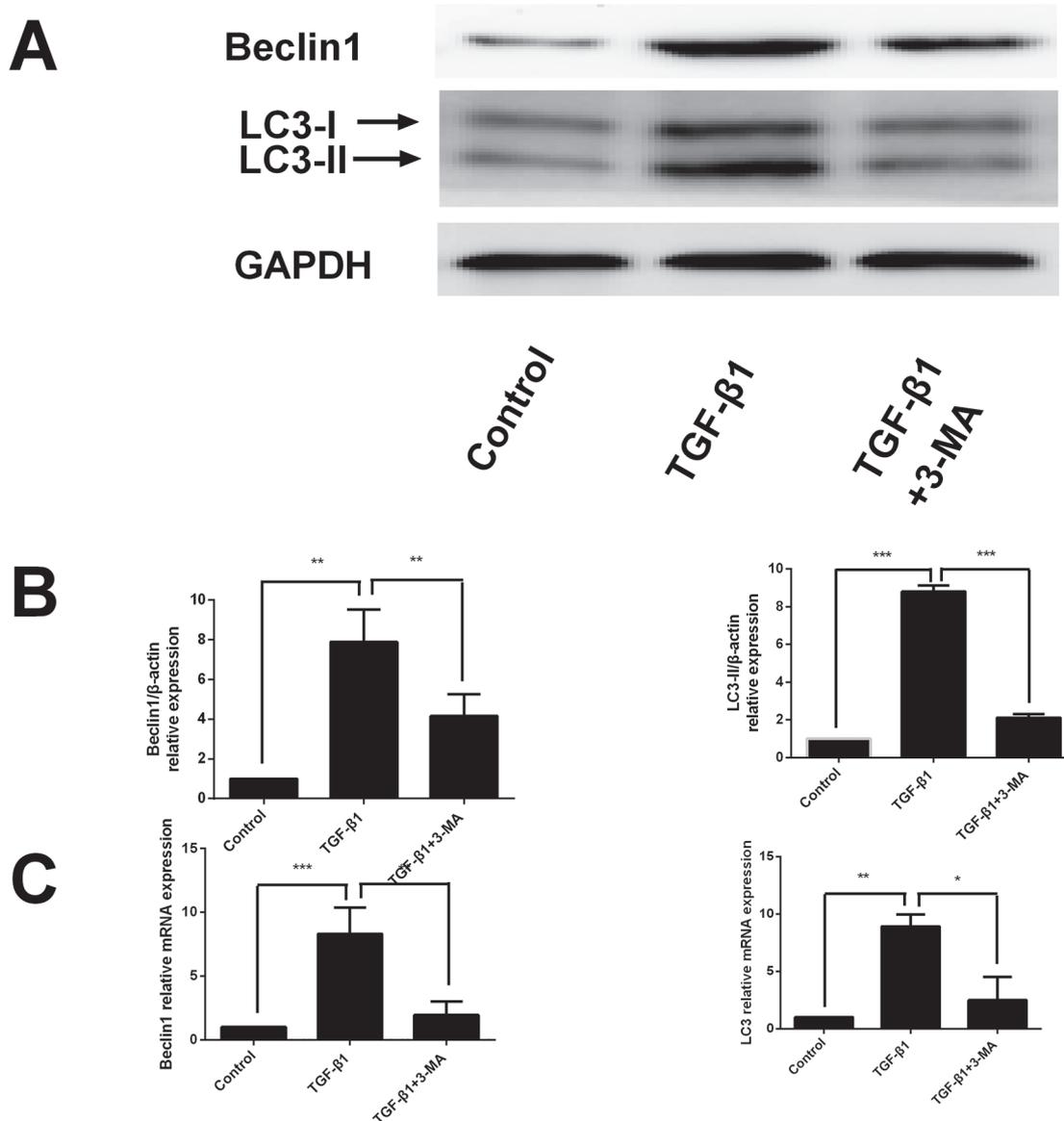
After treatment of TGF- $\beta$ 1 in different concentrations (0 ng/mL, 5 ng/mL and 10 ng/mL) for 24 h, the Western-blot results revealed that after the stimulation of TGF- $\beta$ 1, protein expressions of LC3 and Beclin1, the autophagy-labeled proteins of HpeG2 cell of liver cancer, were elevated with an increase in the concentration of TGF- $\beta$ 1 in a dosage-dependent manner (Figure 1).

### Autophagy Inhibitor-3-MA Blocking the Cell Autophagy Mediated by TGF- $\beta$ 1

After HpeG2 cells were treated using 10 ng/mL TGF- $\beta$ 1 for 24 h, protein expressions of LC3 and Beclin1, autophagy-labeled proteins, were significantly increased compared with the control group. However, autophagy inhibitor-3-MA (5 mmol/L) could inhibit the activity of autophagy (Figure 2).



**Figure 1.** TGF- $\beta$ 1 induces HpeG2 cells autophagy upregulation. **A**, Western blot analysis of Beclin1 and LC3 protein; **B**, Detection of autophagy by using orange acridine staining; **C**, Quantification analysis of Western blot results; **D**, RT-PCR analysis of Beclin1 and LC3 mRNA.



**Figure 2.** Autophagy inhibitor 3-MA inhibits TGF- $\beta$ 1 induced HpeG2 cells autophagy. **A**, Western Blot analysis of Beclin1 and LC3 protein; **B**, Quantification analysis of Western blot results; **C**, RT-PCR analysis of Beclin1 and LC3 mRNA.

### TGF- $\beta$ 1 Promoting HpeG2 Cell Invasion

In the invasion experiment of transwell chamber, we found that after cells were treated using TGF- $\beta$ 1 for 24h, invasion capability of HpeG2 cells in the TGF- $\beta$ 1 treatment group was significantly enhanced in comparison with the control group (Figure 3).

### Inhibition of the Autophagy Weakening the HpeG2 Cell Invasion Mediated by TGF- $\beta$ 1

Further investigations were carried out to verify whether autophagy could affect the cell inva-

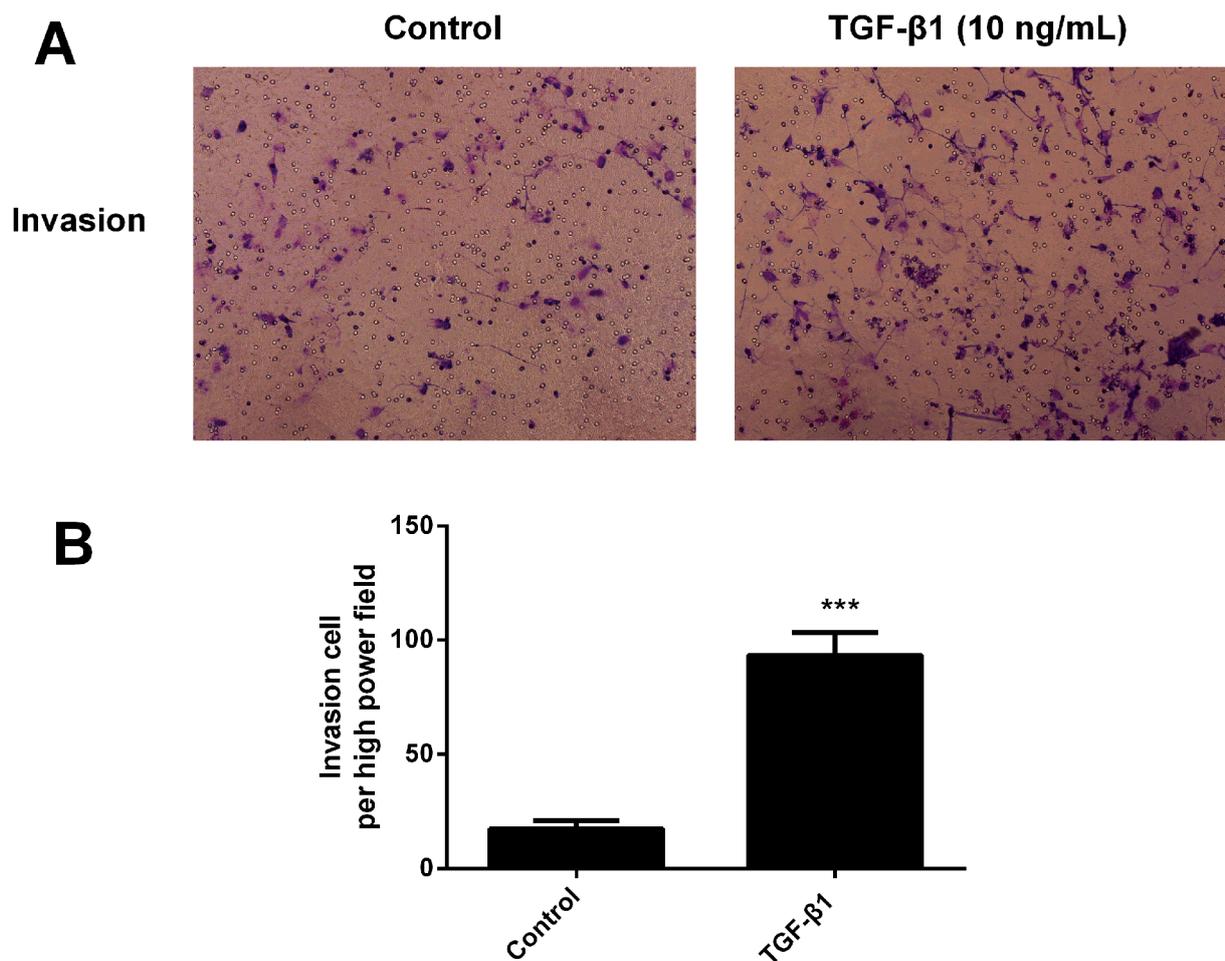
sion mediated by TGF- $\beta$ 1. In the invasion experiment of transwell chamber, we found that after the combined treatment of TGF- $\beta$ 1 and 3-MA (autophagy inhibitor, 5 mmol/L) for blocking the autophagy, the invasion capability of HpeG2 cells was remarkably weakened (Figure 4).

### Discussion

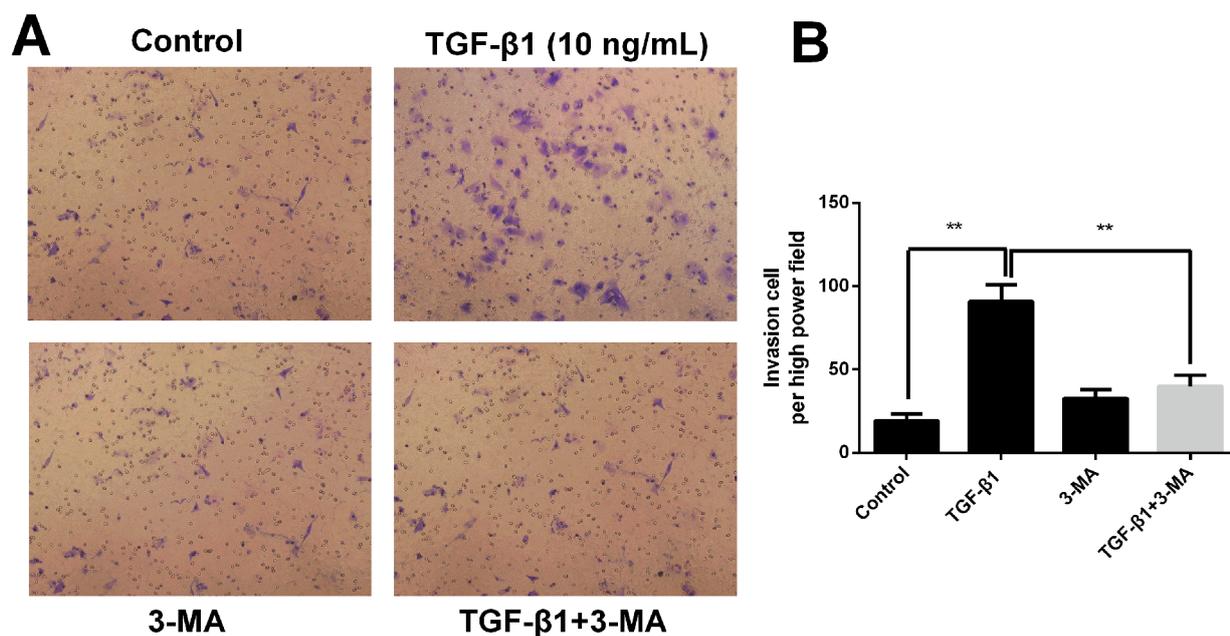
Autophagy, a highly-conservative digestive process that widely exists in the eukaryotes, can degrade the injured mitochondria and pro-

teins inside the cells to release the biomacromolecules, such as amino acid, to provide the nutrition support for cells to sustain the cellular homeostasis. Under the normal circumstance, autophagy remains at a basic level, but when it comes to the metabolic response to stress from the bad environment, like nutrient insufficiency, anoxia, infection, autophagy, as one of the compensatory metabolism of cell in response to the stress condition, can be rapidly activated to sustain the survival of cell in the extreme condition until the stress is fully relieved<sup>9</sup>. More and more studies have shown that autophagy can exert its functions under various pathophysiological condition through regulating cell apoptosis, differentiation, proliferation, growth and development. Also, many literature reported that alterations are found in the levels of autophagic expression in different tumors, which may affect the occurrence and development of

tumors<sup>10</sup>. TGF- $\beta$ , a kind of polypeptide cytokine and a member of TGF- $\beta$  superfamily, participates in a series of biological events of body, including regulating cell growth, differentiation, apoptosis and synthesis of extracellular matrix proteins, and is involved in the inflammations, tissue repair and embryonic development<sup>11</sup>. In the long processes of occurrence and development of malignant tumor, TGF- $\beta$ 1 can exert dual functions depending on the difference in stage and local environment of tumor. In the initial stage of tumor, TGF- $\beta$ 1 can suppress the formation of tumor. However, when it comes to the solid tumor stage, TGF- $\beta$ 1 can promote the local angiogenesis of tumor for nutrient support and promote the local infiltration, distant invasion and metastasis of tumor cells, thus accelerating the growth and formation of tumor. In liver cancer cells, a high-level expression of TGF- $\beta$ 1 can induce the activation of autophagy, resulting in



**Figure 3.** TGF- $\beta$ 1 promotes HpeG2 cells invasion (Coomassie brilliant blue  $\times$  200). **A**, Detection of HpeG2 invasion ability by using transwell invasion assay; **B**, Quantification analysis of transwell assay results.



**Figure 4.** Autophagy inhibitor 3-MA attenuates TGF- $\beta$ 1 induced HpeG2 cell invasion (Coomassie brilliant blue $\times$  200). **A**, Detection of HpeG2 invasion ability by using transwell invasion assay; **B**, Quantification analysis of transwell assay results.

the tolerance of tumor cells to the ischemic and hypoxic microenvironment, thus sustaining the survival of tumor cells<sup>8,12</sup>. Under the relatively rugged environment, mutual regulation between the expression of TGF- $\beta$ 1 and autophagy can guarantee the survival of tumor cells in different stress condition, thus providing the conditions for further invasion and metastasis<sup>13</sup>. In addition, previous studies<sup>14</sup> showed that TGF- $\beta$ 1 can exert key functions in the distant invasion and metastasis of multiple tumors, in which autophagy is also involved. Thus, research into the internal correlation between TGF- $\beta$ 1 and autophagy may illustrate the mechanisms how tumor cells gain the invasion capability and important malignant phenotype. In this study, we established the *in vitro* TGF- $\beta$ 1 model of liver cancer cell to observe the impact of TGF- $\beta$ 1 on autophagic activity and invasion capability of HpeG2 cells to explore the correlation between the invasion capability and autophagy of HpeG2 cells and illustrate the mechanism of TGF- $\beta$ 1 inducing the invasion capability of HpeG2 cells. In this study, we firstly found that under the stimulation of TGF- $\beta$ 1 in different concentrations, transition of autophagy-labeled proteins from LC3 to LC3-I and LC3-II was increased and the expression of Beclin1 was significantly increased in a dosage-dependent manner. This result suggested

that TGF- $\beta$ 1 can sufficiently induce the increase in autophagic activity of liver cancer cells. Furthermore, we used the inhibitor of TGF- $\beta$ 1 to antagonize its impact on the autophagic activity, and found that inhibitor of TGF- $\beta$ 1 can effectively reverse the increase in the autophagic level induced by TGF- $\beta$ 1. We further carried out the transwell invasion experiment to detect the influence of TGF- $\beta$ 1 on invasion capability of liver cancer cells, and the results suggested that invasion capability of liver cancer cells can be enhanced under the stimulation of TGF- $\beta$ 1. Nevertheless, when we used 3-MA, the autophagy inhibitor, to block the autophagy, we found that TGF- $\beta$ 1 failed to increase the invasion capability of liver cancer cells. This showed that autophagy may be an important mechanism for the enhancement in invasion capability of liver cancer cells under the stress conditions. The increase in invasion capability may be another survival mechanism of tumor cells endowed by autophagy. Recently, literature reported that the application of Bafilomycin A1, the autophagic inhibitor, in the advanced stage for inhibiting the autophagic activity can enhance the sensitivity of liver cancer cells to chemotherapy<sup>15</sup>. Also, this effect is reported in the literature on breast cancer and lung cancer<sup>16,17</sup>, providing the potential target for treatment of tumor.

## Conclusions

This work primarily confirmed that TGF- $\beta$ 1 can significantly increase the cell invasion capability of human liver cancer HpeG2 cell strain, and TGF- $\beta$ 1 can regulate the cell invasion capability through upregulating the autophagic expression level. Thus, we will carry out the in-depth study on the roles of autophagy in the occurrence and development of liver cancer and the regulation mechanism of TGF- $\beta$ 1 for autophagy to figure out effective methods to block the cell autophagy and decrease the invasion and metastasis of tumor cells. These studies will provide new ideas and references for specific treatment of liver cancer, which has a wide clinical significance and good prospect of application.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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