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Interleukin-17A inhibits cell autophagy under starvation and promotes cell migration via TAB2/TAB3-p38 mitogen-activated protein kinase pathways in hepatocellular carcinoma

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is characterized by progressive development and poor prognosis against a background of chronic inflammation. Interleukin (IL)-17A is an important proinflammatory cytokine that contributes to inflammatory pathology and tumor microenvironment. Research on autophagy has increasingly focused on its role in inflammation. Thus, we investigated the effect of IL-17A on the progression of HCC through the autophagic pathway.

PATIENTS AND METHODS: The expression and prognostic values of IL-17A and autophagic gene Beclin-1 were determined using immunohistochemistry in 83 HCC patients after resection. The effects and underlying molecular mechanisms of IL-17A on human HCC were explored *in vitro* using recombinant human IL-17A.

RESULTS: High expression of IL-17A and low expression of Beclin-1 were associated with worse TNM stage in HCC patients. And the level of autophagy was lower in tumor tissues compared with tumor-adjacent tissues. *In vitro*, recombinant human IL-17A inhibited starvation-induced autophagy and maintained cell viability through activating TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3)-inducing p38 mitogen-activated protein kinase (MAPK) in Huh7 and HepG2 HCC cells. IL-17A promoted migration of HCC cells through the TAB2/p38 MAPK and TAB3/p38 MAPK pathways.

CONCLUSIONS: IL-17A promotes migration of HCC cells and prevents autophagic cell death from starvation by activating TAB2/p38 MAPK and TAB3/p38 MAPK.

Key words:
Hepatocellular carcinoma, IL-17A, Autophagy, Cell viability, Migration

Introduction

Hepatocellular carcinoma (HCC) has been a major health problem worldwide for a long time. It is the fifth most frequent neoplasm, and the third most common cause of cancer-related death.[1] HCC is short of characteristic diagnostic markers and its prognosis is poor. Recent studies highlight new molecular mechanisms involved in HCC development and progression, including immune cells and their secreted cytokines. They not only disperse cancer cells, but also create a proper microenvironment for tumor development[2]. Despite of recent progress towards understanding the mechanisms underlying HCC tumor formation and pathogenesis, there are still no effective therapeutic methods available[3].

HCC is frequently the long-term result of chronic liver inflammation. A considerable number of studies have demonstrated that innate and adaptive immunity are contributed to the initiation and progression of HCC. Several inflammatory cytokines are currently known to take part in the promotion of HCC. Interleukin (IL)-17A is a proinflammatory cytokine mainly secreted by T helper (Th) 17 cells[4-6]. Cumulative evidences reveal that IL-17 is an essential proinflammatory cytokine, which can induce secretion of many cytokines and chemokines by distinct cell types, such as mesenchymal cells[7]. In addition, IL-17 enhances the expression of antimicrobial peptides that come from epithelial cells and facilitates host defense against infection[8,9]. There are increasing evidences that IL-17 is an important inflammatory cytokine that links innate and adaptive immunity. Moreover, IL-17 has also been frequently detected in many cancers such as gastric cancer[10], breast cancer[11], colorectal cacncer[12] and ovarian cancer[13]. Several studies[14-16] have shown that IL-17 plays a pro-tumor role in HCC and its high expression is associated with poor prognosis. However, the role of IL-17A in the development and progression of HCC remains controversial.

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Autophagy is an important intracellular destructive process leading to the disassemble of the dysfunctional cellular components. Impaired autophagy can induce diverse pathological conditions in humans, ranging from liver dysfunction to tumorigenesis[17]. For instance, compared with adjacent non-tumor tissues, autophagic gene Beclin-1 (Atg6 in yeast) expression is decreased in HCC tissues[18,19]. Suppression of autophagy causes p62 accumulation, as identified in various cancers[20]. Although accumulating evidence indicates that autophagy suppresses tumorigenesis to preserve cellular fitness, its underlying mechanism in carcinogenesis is still apparently complex.

In this study, we aimed to explore whether IL-17A and autophagy can influence the pathophysiological progression of HCC. Also, we hypothesized that there was a strong link between them and clarified the underlying mechanism.

Patients and Methods

Clinical specimens

HCC tissue specimens were obtained from patients after surgical resection in Nanjing Drum Tower Hospital, Nanjing, China. Informed consent was signed by the patients. The patients were selected according to the following criteria: (1) diagnosis of primary HCC; and (2) previously untreated with surgery as the first-line treatment. A total of 83 serum samples of preoperative (2 days) HCC patients were prospectively collected at our hospital, which can reflect the original status of the HCC patients.

Cell lines and reagents

Human HCC cell lines (HepG2, Huh7) were purchased from Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). Both cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). To undertake the stimulation of starvation, all cell lines were maintained in Earle's Balanced Salt Solution for 6 h. Both of the media contained L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were cultured at 37°C in a humidified incubator of 5% CO_2.

Human recombinant IL-17A was purchased from Abcam (Cambridge, UK). Rapamycin was obtained from Sigma (St Louis, MO, USA). The p38 MAPK inhibitor, SB203580 was purchased from Selleck (Houston, TX, USA). DAPI (4',6-diamidino-2-phenylindole) was obtained from Beyotime (Shanghai, China). Anti-human IgG and anti-human IL-17 receptor A (IL-17R), mammalian target of rapamycin (mTOR), anti-IL-17A, p62, LC3B, Beclin-1, T-p38 MAPK, p-p38 MAPK, TAB2, TAB3, BCL-2, caspase 3, Cdc42, Rac and Rho were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell transfection

The targeted siRNAs related to IL-17R, TAB2 and TAB3, as well as negative control mismatch sequences were synthesized by Life Technologies (New York, NY, USA) using the following positive-sense sequence: IL-17R sense, ACG TGG TCC TGA AAT TCG CCC AGTT; TAB2 sense, CCC UAC CUU UGA ACU UAC AAA UCUU; and TAB3 sense, CCC GCA UGG AAG AUG GCU GUU GAGG. Transfection into HCC cells lines was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunohistochemistry

Paraffin-embedded, formalin-fixed HCC tissue sections (5 μm thick) were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min. For antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 10 min in a microwave oven. Non-specific binding was blocked by 5% phosphate buffered saline (BSA) in bovine serum albumin (BSA) for 30 min. The slides were incubated with a 1:100 dilution of antibody against human IL-17A and Beclin-1 at 4°C overnight in a moist chamber. Diaminobenzidine tetrahydrochloride was used as the visualization substrate followed by counterstaining with hematoxylin. Positively stained cells were counted under a microscope by two independent investigators.

Western blotting

Whole-cell lysates from HCC cells were harvested with cell lysis buffer. The homogenates were centrifuged at 12,000 rpm for 10 min and the supernatants were stored at –80°C prior to analysis. Protein concentrations were determined using a bicinchoninic acid (BCA) Protein Assay (Beyotime, Shanghai, China). Protein extract (20 μg) was separated by 10-15% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.05% Tween-20 (TBST), and incubated with the

primary antibodies, described previously, overnight at 4°C. The membrane was then incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody at room temperature, and developed with the electrochemiluminescence (ECL) plus kit (Millipore, Billerica, MA, USA) after washing with TBST.

Cell viability

Cell viability was measured using the CCK-8 assay (Dojindo, Shanghai, China). About 3000 cells were plated in each well of 96-well plates at 37°C in a humidified 5% CO_2 atmosphere for 12 h before being treated with the indicated compounds. After treatment, 10 µl CCK-8 was added to each well, and the cultures were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The medium was measured with a Varioskan Flash reader (Thermo Fisher, Waltham, MA, USA) at 490 nm. Each sample was repeated three times for standard deviation calculations.

Immunofluorescence staining

For immunofluorescence staining, the monoclonal antibody LC3B and DAPI were used. HCC cells were plated on glass-bottom dishes and treated with or without the indicated agents. The cells on the dishes were washed twice with PBS slightly, fixed in 4% paraformaldehyde for 30 min, and washed three times after fixation. The cells on the dishes and HCC sections (5 mm thick) were prepared and stained with the indicated primary antibody (LC3B) overnight at 4°C. The cells and sections were washed twice, incubated with fluorochrome-labeled secondary antibody (1:1000) for 90 min, and washed three times after staining. The cells and sections were incubated with DAPI (1:15, diluted with carbinol) for 15 min at 37°C, and then washed twice with carbinol. Images were obtained with a Zeiss (Jena, Germany) immunofluorescence microscope. The autophagosomes were identified by LC3 dots. Apoptosis was identified by the morphology of cells stained with DAPI.

Migration assay

A 8-µm-pore-fiter insert (Becton Dickinson, Franklin Lakes, NJ, USA) was used in the migration assay. HCC cells (1×10^5cells/chamber) were added in the upper chamber maintained at 37°C migrated for 48h. The cells on the membrane were washed twice, fixed in 4% paraformaldehyde for 30 min, and washed three times after fixation. Then, the cells were immersed in carbinol for 20 min. The membranes were stained with 1% crystal violet (diluted by carbinol). Migration was assessed by counting the number of stained cells from 6 random fields at × 200 magnification.

Wound healing assay

HepG2 cells were seeded in 6-well plates and cultured until confluence. A wound was, then, created by manually scraping the cell monolayer with a 200-microliter pipette tip. The cultures were washed twice with serum free medium to remove floating cells. The cells were then incubated in DMEM supplemented with 1% FBS. Cell migration into the wound was observed at 12 h in eight randomly selected microscopic fields for each condition and time point. Images were acquired with a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope (Wetzlar, Germany).

Statistical Analysis

All data were analyzed with SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and $p<0.05$ was considered statistically significant. The Student *t*-test was used as appropriate for the comparison of variables. Associations between each marker and clinical characteristics were evaluated using Pearson's χ^2 test.

Results

High expression of IL-17A and low expression Beclin-1 in HCC tissue

As shown in Figure 1A, IL-17A could be only detected in a part of patient tumor tissues, which showed staining patterns surrounding the lymphocytes. Expression in HCC tissues was more strongly positive than in none-tumor tissues. Coincidently, the high expression of IL-17A was paralleled with low expression of Beclin-1 in HCC tumor tissue (Figure 1A). Tumor tissues that expressed low levels of IL-17A also showed high expression of Beclin-1 (Figure 1A and B). All non-tumor tissues also showed high expression of Beclin-1.

Autophagy was inhibited in HCC tissue

Abnormal expression of Beclin-1 was indicative of changes in autophagy in HCC. Protein expression of autophagic genes (mTOR and p62) was detected. In IL-17A positive tumor tissue, inhibitory autophagic protein expression of mTOR and p62 was higher in tumor tissue than in none-tissue. By contrast, there was no difference between tumor and none-tumor tissue in IL-17A negative tumor tissue (Figure 1A and B). The same results were

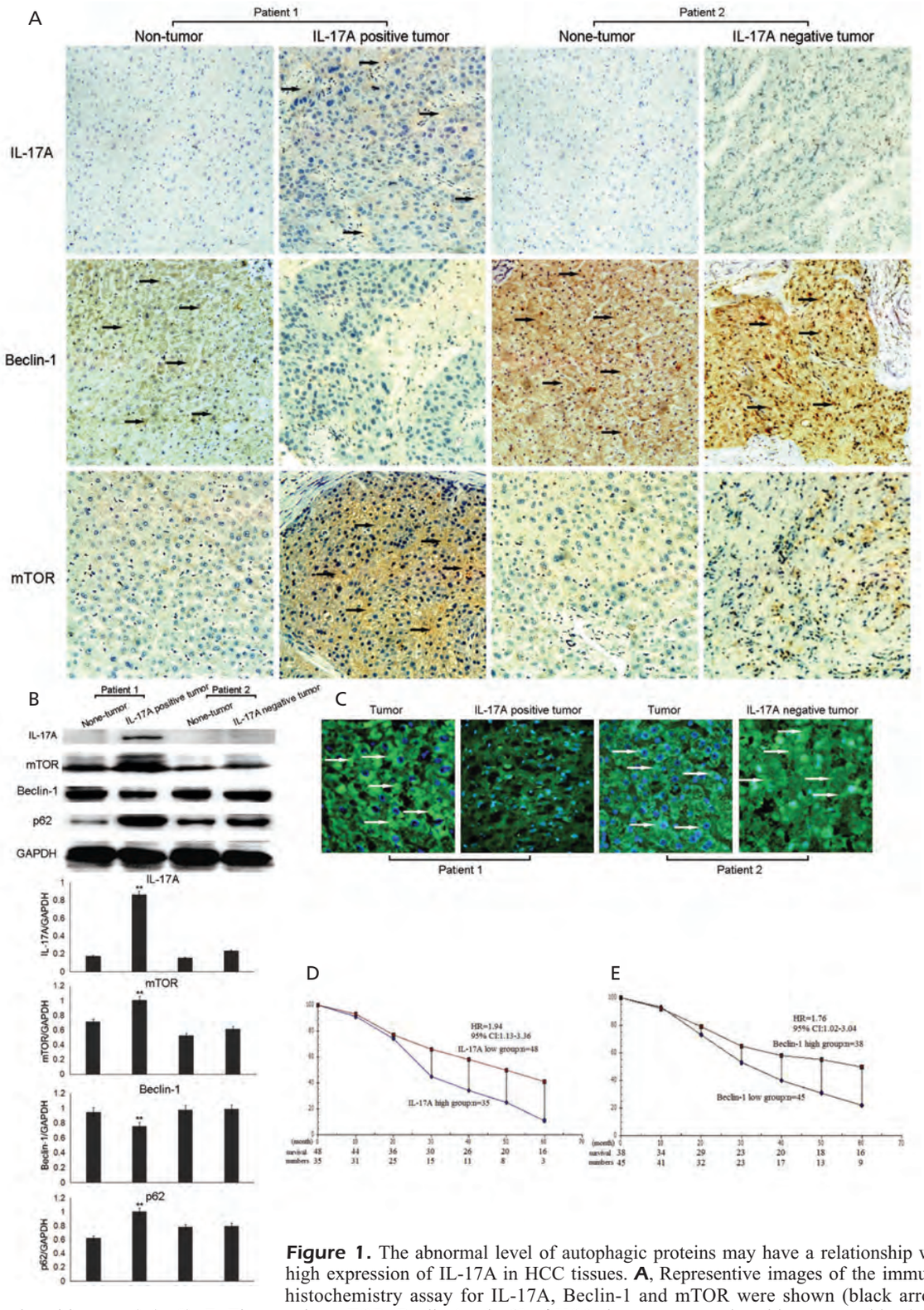

Figure 1. The abnormal level of autophagic proteins may have a relationship with high expression of IL-17A in HCC tissues. **A**, Representive images of the immunohistochemistry assay for IL-17A, Beclin-1 and mTOR were shown (black arrows showed positive areas) (n=3). **B**, The proteins mTOR, Beclin-1and p62 of HCC tissues were analyzed by western blot (n=3). **C**, LC3B foci (white arrows showed LC3B dots) in HCC cells were detected by immunofluorescence (n=3). **D**, The relationship between the overall survival and IL-17A in HCC patients was shown (n=3). **E**, The relationship between the overall survival and Beclin-1 in HCC patients was shown. (n=3).

detected by LC3B immunofluorescence. When compared with non-tumor tissue and IL-17A negative tumor tissue, the LC3 foci were fewer than in IL-17A positive tumor tissue (Figure 1C). These data indicated that the abnormal level of autophagy may have a relationship with high expression of IL-17A in HCC, and that its disturbance might contribute to the progression of HCC.

IL-17A and Beclin-1 were associated with poor prognosis of HCC

Previous studies have verified that abnormal expression of IL-17A and Beclin-1 has a close relationship in cancer patients separately. Thus, we next retrospectively investigated the whether there is a correlation between IL-17A and Beclin-1 in tumor tissue with clinicopathological features of 83 HCC patients. HCC cases were divided into four groups: IL-17A-high groups (above the mean level, n=35); IL-17A-low groups (below the mean level, n=48), Beclin-1-high groups (n=38) and Beclin-1-low groups (n=45). An associated study showed that the frequency of IL-17A in tumor tissue was not significantly associated with gender, age, α-fetoprotein (AFP), history of hepatitis, cirrhosis, and tumor number, size and differentiation. However, high expression of IL-17A in tumor tissue was associated with TNM stage (p=0.018) (Table I) and overall survival rate (Figure 1 D). It is noteworthy that low frequency of Beclin-1 in tumor tissue had a similar relationship with TNM stage as in the IL-17A-high group (Table I). Once more, owing to the important role of Beclin-1 in autophagy, we had reason to speculate IL-17A could regulate HCC development through autophagy.

IL-17A decreased autophagy in HCC cells under the condition of starvation

To determine whether the decreased autophagy was induced by IL-17A in HCC, we examined whether IL-17A could directly regulate autophagy in cultured Huh7 and HepG2 cells. In according with our hypothesis, IL-17A significantly inhibited the starvation-induced increase of Beclin-1 in HCC cells at 30 ng/ml in a dose-dependent manner (Figure 2A). In addition, IL-17A inhibited the formation of the LC3 foci (Figure 2C), reduced the ratio of LC3-II/LC3-I (Figure 2B), and enhanced p62 and mTOR in Huh7 and HepG2 cells (Figure 2B). To further demonstrate the role of IL-17A in HCC, IL-17R expression was reduced by siRNA. In HCC cells exposed to IL-17R-targeted siRNA (HCC-siRNA-IL-17R), IL-17A inhibition of autophagy was significantly enhanced (Figure 3). These data indicate that IL-17A directly inhibits autophagy in HCC cells under the condition of starvation.

Table I. Correlation of IL-17A and Beclin-1 with clinicopathological features in 83 HCC patients.

Characteristics		IL-17A level			Beclin-1 level		
		Low	High	P	Low	High	P
Gender	Male	40	29	0.059	36	33	0.298
	Female	8	6		9	5	
Age (years)	≤53	8	30	0.459	23	25	0.060
	>53	8	37		10	25	
AFP (μg/L)	≤50	29	24	0.316	6	25	0.190
	>50	19	11		16	36	
Hepatitis history	Yes	36	24	0.344	20	47	0.480
	No	12	11		4	12	
Cirrhosis	Yes	42	32	0.423	21	46	0.250
	No	6	3		3	13	
Tumor nodule (n)	Single	43	30	0.614	14	45	0.263
	Multiple	6	4		8	16	
Tumor size (cm)	≤5	33	20	0.196	21	23	0.203
	>5	15	15		22	15	
Differentiation	Well and moderatied	35	25	0.584	4	6	0.294
	poorly	13	10		40	33	
TNM stage	I	36	20	0.003	7	33	0.038
	II-III	10	17		16	27	

AFP: α-fetoprotein

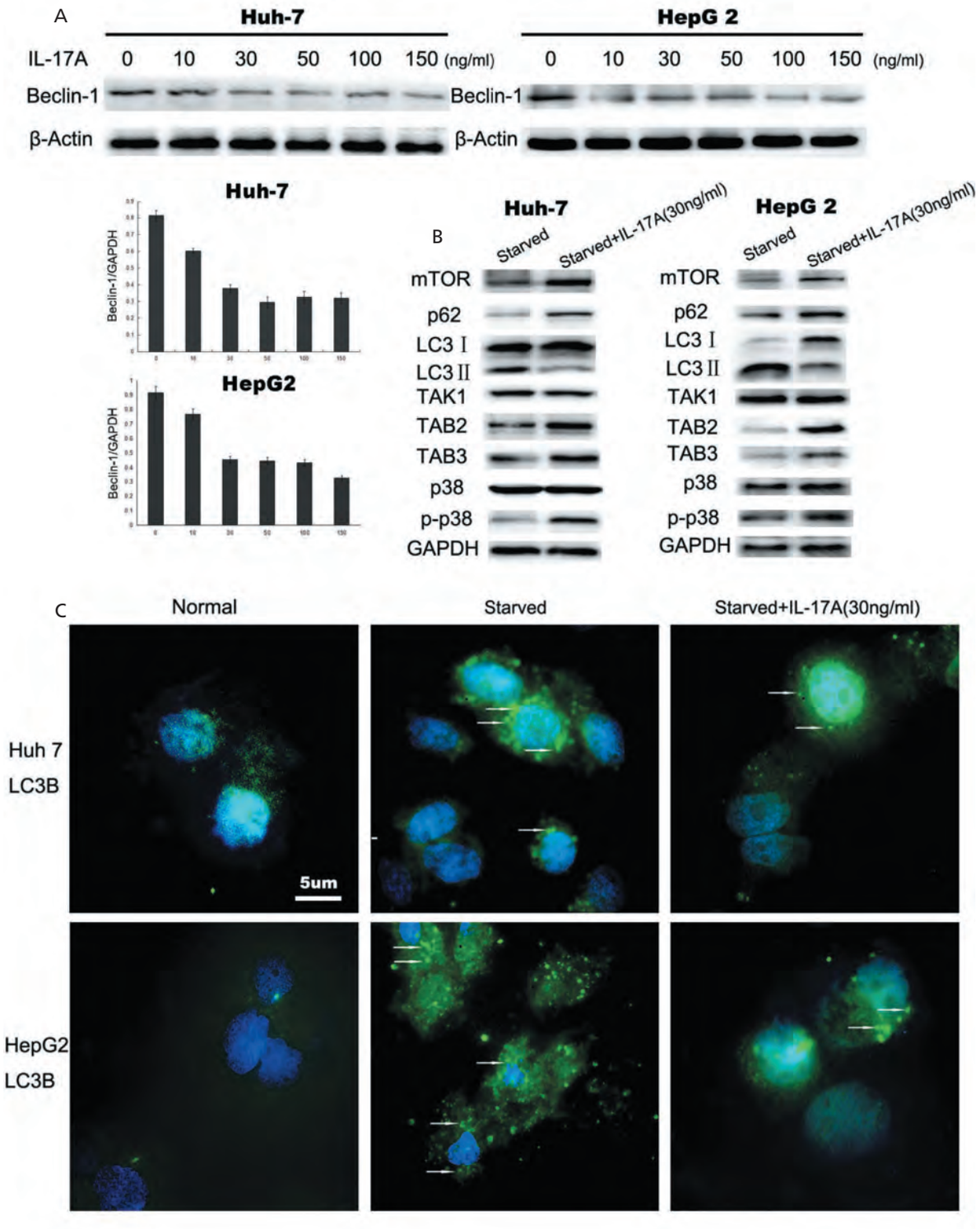

Figure 2. IL-17A inhibited autophagy in HCC cells. **A**, The HCC cells were treated by 0, 10, 30, 50 or 100 ng/ml of IL-17A. The expression of Beclin-1 was detected by Western blot analysis. (n=3) **B**, HCC cells were treated with or without 30 ng/ml of IL-17A under the condition of starvation Western blotting analyzed the expression of mTOR, p62, LC3-I, LC3-II, TAK1, TAB2, TAB3, p38, p-p38. GAPDH was as control (n=3) that IL-17A inhibited starvation-induced autophagy. At the same time, IL-17A enhanced expression of TAB2 and TAB3, activated the p38 MAPK pathway. However, there was no effect on TAK1. **C**, HCC cells were treated with or without 30 ng/ml of IL-17A under the condition of starvation. The HCC cells under normal condition without 30 ng/ml of IL-17A were as control. LC3B foci (white arrows showed LC3B dots) in HCC cells were detected by immunofluorescence (n=3).

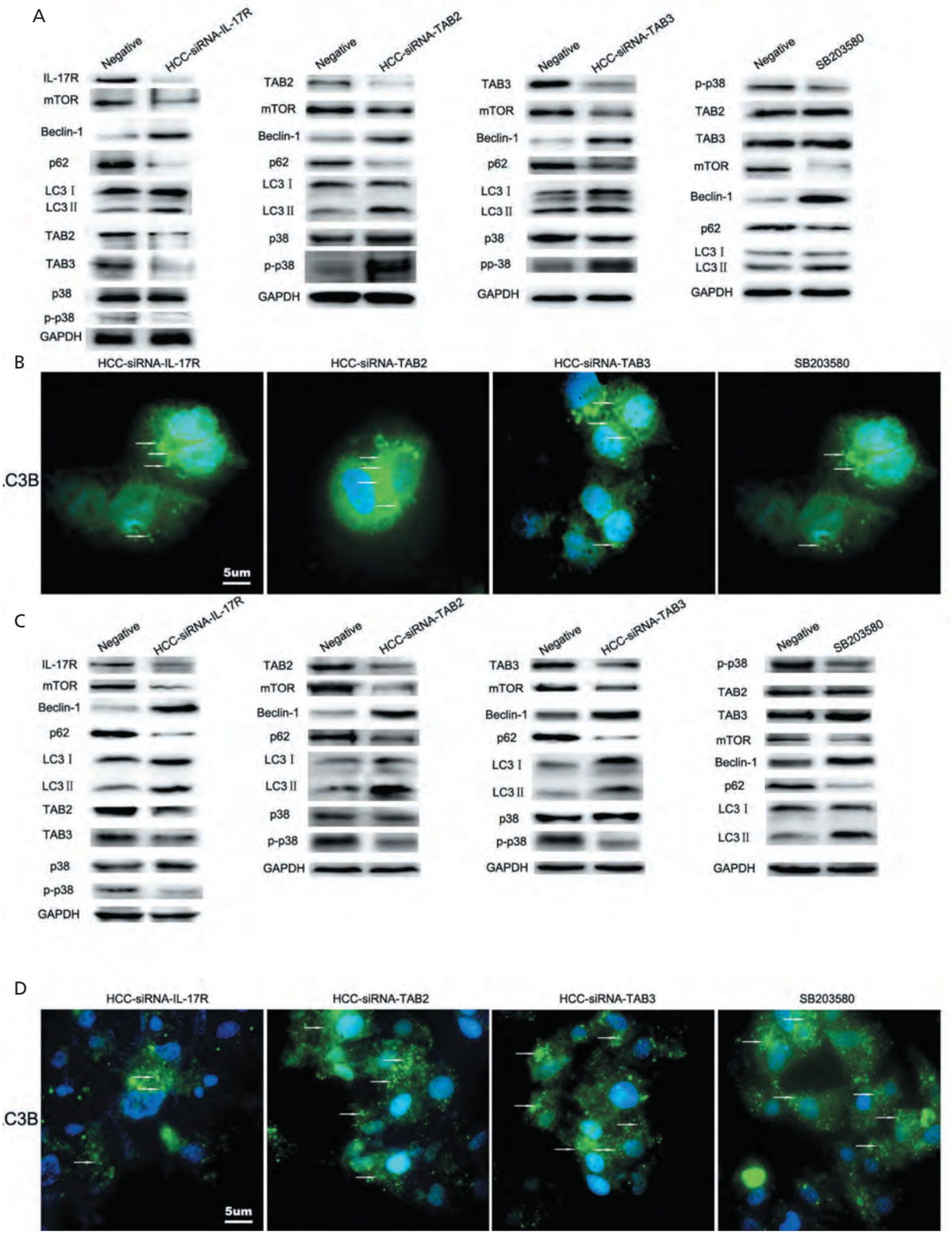

Figure 3. Transient knockdown of IL-17R, TAB2 and TAB3 and cells cultured with SB203580 reversed effects of IL-17A.

IL-17A regulated autophagy through the p38 MAPK signaling pathway under the condition of starvation

Various signaling pathways are suggested to mediate IL-17A action. Because p38 mitogen-activated protein kinase (MAPK) also regulates the autophagy[24], we examined whether IL-17A regulated autophagy through the p38 MAPK signaling pathway under the condition of starvation. There was no significant change in total expression of p38 MAPK in Huh7 and HepG2 cells. However, phosphorylation of p38 MAPK was increased after IL-17 treatment for 6 h (Figure 2B). When the HCC-siRNA-IL-17R was added to the culture medium, the IL-17-inducing activation of p38 MAPK was significantly diminished (Figure 3). Given that autophagy was decreased in parallel with p38 MAPK activation in HCC cells treated with IL-17A, p38 MAPK signaling may play an important role in inhibiting autophagy and, hence, tumor progression.

To verify the potential mechanism of p38 MAPK-mediated effects on HCC, the p38 MAPK inhibitor SB203580 was used to reduce activation of p38 MAPK. In HCC cells exposed to SB203580, IL-17A-induced p38 MAPK was significantly reduced (Figure 3A and 3C), and the expression of autophagy-related protein (LC3B, Beclin-1, mTOR and p62) was significantly reversed (Figure 3A and 3C). Similar results appeared for LC3B immunofluorescence foci (Figure 3B and 3D). These results demonstrated that inhibition of autophagy by IL-17A in HCC cells was via activation of p38 MAPK signaling pathway.

TAB2 and TAB3 mediated downregulation of autophagy

Since the expression of Transforming Growth factor-β (TGF-β) activated kinase (TAK1) can be enhanced by IL-17A[21] and TAK1-binding protein (mainly TAB2 and TAB3) functioned as tonic inhibitors of autophagy[22,23], we next investigated whether TAK1, TAB2 and TAB3 mediated the function of IL-17A in HCC autophagy. Indeed, IL-17A enhanced expression of TAB2 and TAB3 (Figure 2B). However, there is no change in expression of TAK1. When cells were cultured with HCC-siRNA-IL-17R, expression of TAB2 and TAB3 decreased (Figure 3A and 3B). As TAB2 and TAB3 are negative regulators of autophagy, we next examined whether IL-17A inhibition of autophagy depended on the presence of TAB2 and TAB3. Indeed, disturbing TAB2 and TAB3 with siRNA (HCC-siRNA-TAB3 and HCC-siRNA-TAB2) partly reversed the IL-17A-inhibited autophagic protein expression (Figure 3A and 3C). As for the cells cultured with HCC-siRNA-TAB2 or HCC-siRNA-TAB3, the number of LC3B foci was increased in parallel (Figure 3B and 3D). These results indicated that IL-17A inhibits the autophagic process in HCC cells in a TAB2- and TAB3-dependent manner.

The p38 MAPK signaling pathway also has a close relationship with TABs[25]. Therefore, we investigated the role of TAB2 and TAB3 in p38 MAPK activation in IL-17A-stimulated HCC cells. Western blotting found that IL-17A-induced p38 MAPK activation was inhibited in cells treated with HCC-siRNA-TAB2 or HCC-siRNA-TAB3 (Figure 3A-C). However, there was no change in expression of TAB2 and TAB3 in cells cultured with SB203580 (Figure 3A and C). This suggests that TAB2 and TAB3 mediate the regulation of IL-17A-stimulated p38 MAPK activation and, thus, inhibit the autophagy process.

IL-17A maintained the cell viability of HCC cells under starvation

Some studies have shown that recombinant human IL-17A does not promote HCC cell growth directly, but none has shown whether IL-17A plays a direct role in HCC cells under starvation. Cell viability assay was used to study the effect of IL-17A on activity of Huh-7 cells. CCK-8 assay showed that cell viability rate was increased in HCC cells under 6 h starvation with IL-17A (Figure 4A and 4C). When HCC-siRNA-IL-17R, HCC-siRNA-TAB2, HCC-siRNA-TAB3, SB203580 and Rapamycin (a mTOR inhibitor and stimulator of autophagy) were added to cell culture medium before IL-17A treatment, cell viability rate was decreased (Figure 4B and 4D). The results show that IL-17A maintained the viability of HCC cells under starvation.

IL-17A inhibited apoptosis of HCC cells

To observe the morphological changes of HCC cells incubated with IL-17A under the condition of starvation, cells were stained with DAPI and examined by bright-field and fluorescence microscopy. IL-17A-treated cells had less nuclear condensation, membrane blebbing, were shrunken compared with control cells (Figure 4G). Western blotting showed that Bcl-2 and caspase 3 were markedly changed under stimulation with IL-17A (Figure 4E and 4F). By contrast, in cells treated with HCC-siRNA-IL-17R, HCC-siRNA-TAB2 or HCC-siRNA-TAB3, IL-17A-induced prevention of apoptosis was significantly blocked (Figure 4E-4G).

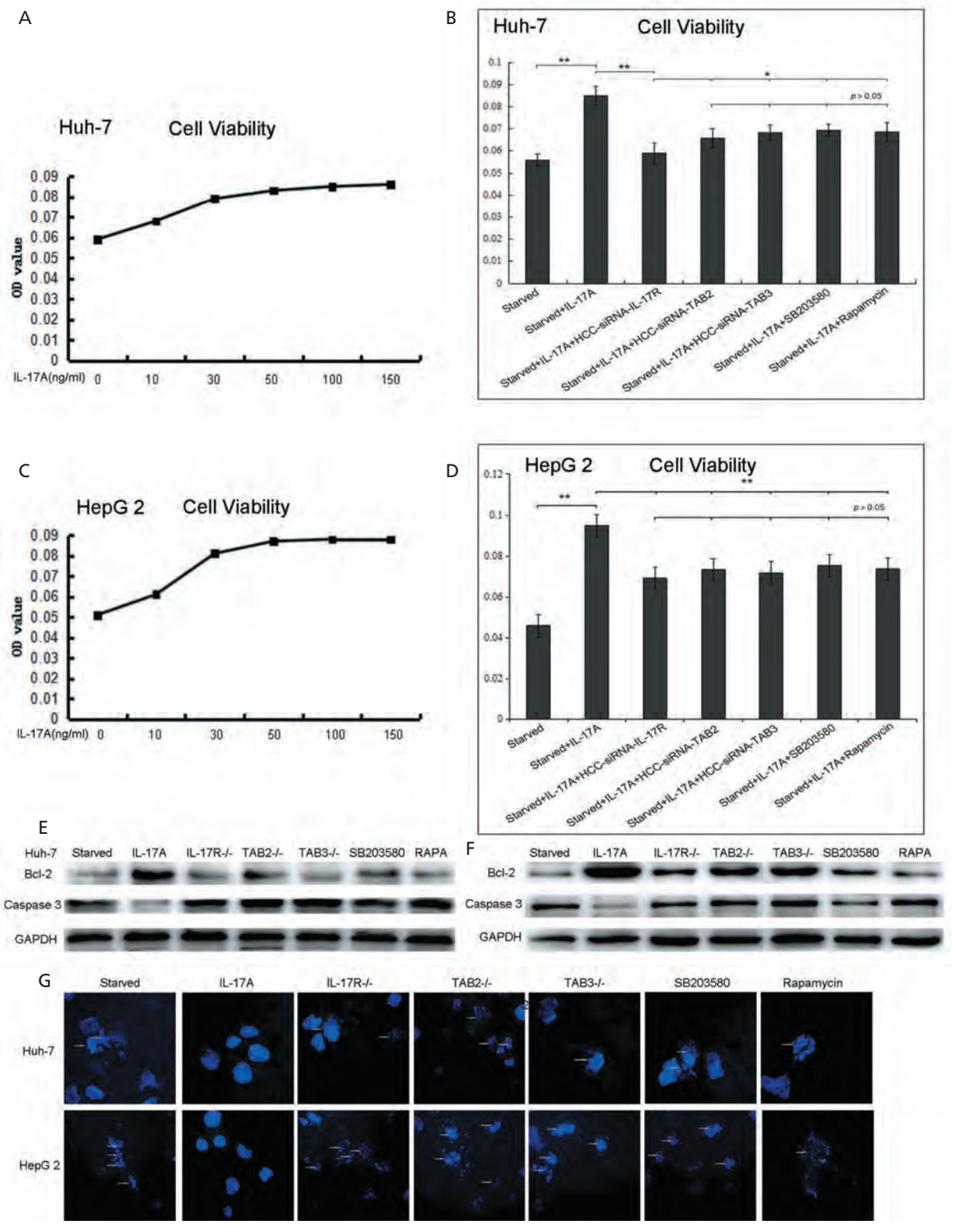

Figure 4. IL-17A maintained cell viability and inhibited cell apoptosis cell death under condition of starvation through TAB2/p38 MAPK and TAB3/p38 MAPK pathways. **A**-**D**, Cell viability was tested by CCK-8 assay. IL-17A maintained cell viability in a dose-dependent manner. Transient knockdown of IL-17R, TAB2, and TAB3 or cultured with SB203580 inhibited the effects of IL-17A. **E** and **F**, Bcl-2 and caspase 3 were investigated by western blotting and the morphological changes in the nuclei were detected by DAPI staining.

As the same, cells cultured with SB203580 or rapamycin reversed the apoptosis level induced by IL-17A stimulation in HCC cells (Figure 4E-4G). These data indicated that autophagy was impaired in IL-17A-challenged HCC cells, and blocking IL-17A and its downstream proteins (TAB2, TAB3 and p38 MAPK) can induce autophagy and its associated cell death in HCC cells.

IL-17A promoted HCC migration through TAB2 and TAB3

IL-17A is significantly elevated in HCC patients and correlated with poor survival, and has a close relationship with tumor metastasis through IL-17R signaling pathways[26]. However, the underlined mechanism was still vague. Thus, we investigated whether IL-17A controlled migration of HCC cells through TAB2 or TAB3. The RhoGTP kinases (Cdc42, Rac and Rho) were enhanced after the stimulation of IL-17A. Disturbing the expression of IL-17R, TAB2, TAB3 and p-p38 MAPK, the expression of Cdc42, Rac and Rho was reversed (Figure 5A). Wound healing assay revealed the similar results in the wound closure rates after IL-17 stimulation for 24 h (Figure 4B). The rates were reversed in cells cultured with HCC-siRNA-IL-17R, HCC-siRNA-TAB2, HCC-siRNA-TAB3 and SB203580 (Figure 4B). The Transwell assays also showed the resemble results (Figure 5C). These data indicate that IL-17 can promote migration of HCC through TAB2/TAB3-p38 MAPK pathways *in vitro*.

Discussion

As one of the highest rates of cancer-related mortality worldwide, HCC has a high recurrence rate after curative therapy and lacks biomarkers for early detection[26]. Some studies have shown that HCC mainly develops from chronic inflammatory disease[27,28], in which many inflammatory cytokines infiltrate the tumor tissues. IL-17A is an important inflammatory cytokine and its expression is higher in tumor tissues than normal tissues[10-11,29-31]. Several recent studies[32,33] showed that $CD8^+$T cells expressed Th17 and IL-17A were attracted to HCC tissues and increased in number in the local tumor environment. The increased intratumoral IL-17-producing cells was negatively correlated with survival in HCC patients[34], while the complex relationship between IL-17A and HCC progression are still gloomy. The effect of IL-17A on cancer progression has been addressed but the results are controversial. On the one hand, some reports[35,36] showed that IL-17A inhibited tumor growth and metastasis via activation of cytotoxic T cells or induction of interferon-γ expression. Moreover, other reports demonstrated that IL-17A promoted metastasis via affecting the tumor microenvironment[37,38], or inducing expression of other cytokines that promote tumor development[34,39].

In this study, we demonstrated that IL-17A played a crucial role in the progression of HCC. We observed the high expression of IL-17A in a part of HCC tissues using immunohistochemistry. Remarkably, patients with high expression of IL-17A had worse TNM stages and lower overall survival rate than those with low expression. These findings indicated that intratumoral IL-17A was involved in the regulation of HCC progression. Therefore, we assumed that IL-17A might act as a novel prognosticator for poor outcome of HCC patients after surgery. In the present study, we found that the expression of autophagic gene Beclin-1 was low in HCC patients, which was negatively associated with prognosis of HCC, suggesting that Beclin-1 also played an important role in promoting HCC progression. Moreover, we detected the expression of mTOR and p62 and found that it was higher in in IL-17A positive tumor tissue. These data indicated that the abnormal level of autophagy may have a relationship with high expression of IL-17A in HCC, and that its disturbance might contribute to the progression of HCC.

We speculated that IL-17A could regulate HCC progression through autophagy. We observed that IL-17A not only inhibited the level of autophagy, but also attenuated autophagy-regulated cell death in cultured HCC cells under starvation. Transient interference with the expression of IL-17R elevated the level of autophagy, decreased HCC cell viability, and induced activation of apoptosis. Moreover, the autophagy activator rapamycin restored the IL-17A-suppressed autophagy and autophagy-associated cell death. Thus, our data demonstrated that IL-17A directly inhibited autophagy in HCC cells under the condition of starvation and inhibition of IL-17A secretion may provide a new treatment strategy against HCC.

TAK1 is an established downstream factor in many cytokines, including IL-17, tumor necrosis factor, transforming growth factor β and Toll-like receptors. TAK1 is involved in regulating cell survival and proliferation, inflammation, and tumorigenesis.

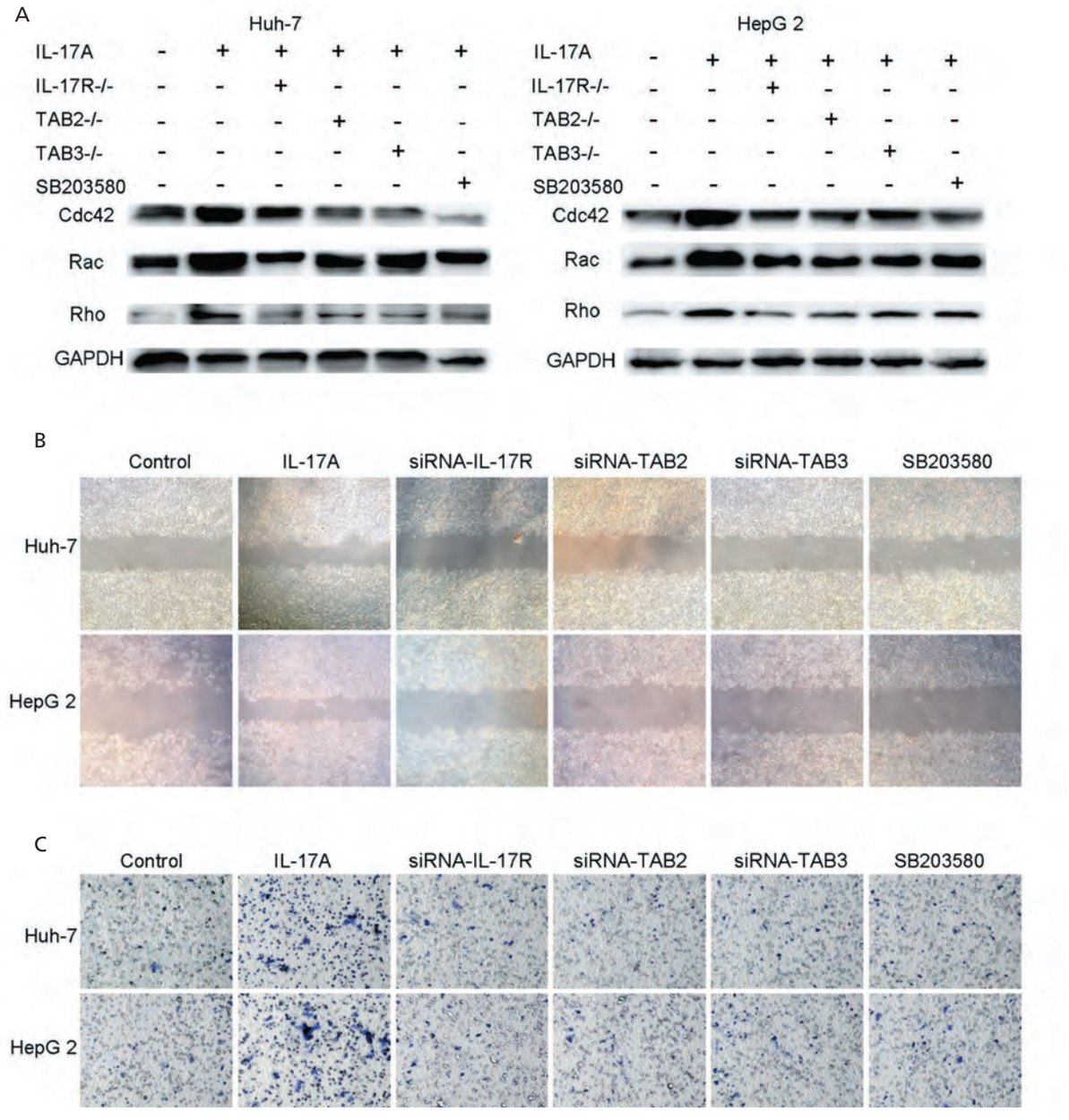

Figure 5. IL-17A promoted cell migration in normal culture condition. **A**, IL-17A increased the expression of RhoGTP kinases through TAB2/ TAB3-p38 MAPK pathways. **B** and **C**, Wound healing assays and Transwell assays were used to prove the effect of IL-17A on HCC cell migration.

However, there was no significant change in expression of TAK1 in HCC cells cultured with IL-17A. To our surprise, the expression of TAB2 and TAB3, two TAK1-binding proteins that are autophagy inhibitors, was increased when IL-17A was added to the culture medium. Transient interference with the expression of TAB2 and TAB3 partly reversed the autophagy-inhibitory effects of IL-17A, thus influencing the viability of HCC cells under starvation. These observations indicated that TAB2 and TAB3 are stimulated by IL-17A, and high expression of TAB2 and TAB3 could inhibit the autophagy induced by starvation in HCC cells.

IL-17A changes cellular activities through activating a variety of signal transduction pathways, such as the MAPKs[39]. Regulation of related gene transcription and changing cell stability

are major mechanisms for the IL-17A-actived signaling pathways and cellular biological functions. The p38 MAPK pathway is activated in response to inflammatory cytokines or a variety of cellular stresses and plays a role in apoptosis and cell cycle regulation.[41] Some studies have shown that activation of p38 MAPK can inhibit autophagy.[42] Indeed, our data showed that IL-17A inhibited autophagy through activation of p38 MAPK. When cells were cultured with IL-17A under starvation, the phosphorylation level of p38 MAPK was elevated. Transient disruption of IL-17R, TAB2 and TAB3 further demonstrated that phosphorylation of p38 MAPK was significantly correlated with IL-17A and its downstream proteins TAB2 and TAB3. The inhibition of autophagy by IL-17A was inhibited by p38 MAPK inhibitor, suggesting that the downregulating role of IL-17A in autophagy might be through activation of p38 MAPK.

Li et al[14] have demonstrated that IL-17A promotes HCC metastasis, but the underlying molecular mechanisms have not been clarified. Thus, we examined whether TAB2, TAB3, p38 MAPK and autophagy were involved in the regulation of metastasis by IL-17A. We showed that IL-17A could enhance migration of HCC cells, which was reversed by HCC-siRNA-IL-17R, HCC-siRNA-TAB2, HCC-siRNA-TAB3, SB203580 and rapamycin. Therefore, it indicated that IL-17 can promote migration of HCC through TAB2/TAB3-p38 MAPK pathways.

However, there are some disadvantages in our study. We used immunohistochemical staining only to identify Beclin-1, as it could not fully reveal the changes in autophagy level. The samples of HCC tissues were not enough, which could led to the selection bias. In addition, we only studied IL-17R, and whether other IL-17R families were involved in the regulation of autophagy was not determined. In response to stimulation by IL-17A, the roles of TAB2 and TAB3 might be different. Various synergistic effects from other proteins that are activated by IL-17A may participate in this process. We consider that there are more linkages between IL-17A and autophagy than those revealed in this study. Therefore, extensive studies are needed in the future.

Conclusions

We showed that high expression of IL-17A and autophagy-related gene Beclin-1 was associated with poor clinical outcome of HCC after tumor resection. *In vitro*, IL-17A inhibited autophagy through phosphorylation of p38 MAPK by elevating expression of TAB2 and TAB3, and then increased the toleration of HCC cells to starvation, and decreased the level of apoptosis. In summary, tumor promoter IL-17A may be a useful predictor for HCC patients survival after resection and a possible therapeutic target against this disease.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Competing interests

The authors declare that they have no competing interests.

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