Original Paper

IL-17 Activates the IL-6/STAT3 Signal Pathway in the Proliferation of Hepatitis B Virus-Related Hepatocellular Carcinoma

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Key Words

IL-17 • IL-6 • pSTAT3 • HBV • HCC

Abstract

Background/Aims: We performed this study to determine the role of IL-17 in the immune microenvironment of hepatitis B virus- (HBV-) related hepatocellular carcinoma (HCC). **Methods:** HepG2 cells were treated with IL-17, STAT3 inhibitor S31-201 or IL-6 neutralizing monoclonal antibody (IL-6 mAb). Cell proliferation and migration were compared using the Cell Counting kit-8 (CCK-8) and Transwell assays, respectively. Real-time quantitative PCR (RT-qPCR), Western Blot, ELISA, immunofluorescence and histological staining were used for determining the expression levels of IL-17, IL-6, MCP-1, CCL5, VEGF, STAT3 and p-STAT3. HCC xenograft models were constructed in wild type and IL-17 knockout mice to clarify the effects of IL-17 on HCC *in vivo*. **Results:** Exogenous IL-17 enhanced the proliferation and migration of HepG2 cells, and it activated the phosphorylation of STAT3. RT-qPCR and ELISA showed that IL-17 promoted the expression of IL-6. The CCK-8 and Transwell assays showed that S31-201 or IL-6 mAb remarkably reversed the promotion effects of proliferation and migration by exogenous IL-17 in HepG2 cells. Additionally, IL-6 could promote the phosphorylation of STAT3, while IL-6 mAb acted as an inhibitor, and exogenous IL-17 could neutralize the inhibitory effects of IL-6 mAb. *In vivo*, compared to the wild type mice, the tumor volume, weight, density and size were decreased in IL-17 knockout mice. Additionally, the expression levels of p-STAT3, IL-6, MCP-1, CCL5 and VEGF decreased in IL-17 knockout mice. **Conclusions:** IL-17 can enhance the proliferation of HepG2 cells *in vitro* and *in vivo* via activating the IL-6/STAT3 pathway. Therefore, the IL-17/IL-6/STAT3 signaling pathway is a potential therapeutic target for HBV-related HCC.

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Introduction

Hepatocellular carcinoma (HCC), the third most prevalent cause of cancer-related death across the world, is one of the five most common cancers [1]. Approximately 20,000 estimated new HCC cases in the United States were reported in 2010 [2]. A number of studies showed that hepatitis B virus (HBV) infection, excess alcohol consumption, exposure

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to aflatoxin and smoking are the major risk factors of HCC [3], and persistent HBV infection is the most dangerous and prevalent cause [4]. Hepatic carcinogenesis, triggered by HBV, involves complicated mechanisms, which are related to immune responses induced by HBV infection and replication, such as inflammatory cytokine expression [5].

Variation in the clinical outcomes of HBV infection and the disease course of HCC may result from numerous immuno-inflammatory cells, cytokines and chemokines [6]. Among the inflammatory signaling pathways that are related to tumor development, the phosphorylation of signal transducer and activator of transcription (STAT3) plays a key role [7]. STAT3, an oncogenic transcription factor, is usually constitutively active in many human cancers, such as prostate cancer, breast cancer and several other malignancies [8-10]. STAT3 has been considered a target for anti-cancer therapy because it plays an important role in tumor cell survival and proliferation [11]. The STAT3 molecule is also responsible for anti-apoptosis activity and cell proliferation of HCC cells [12]. Additionally, STAT3 can be activated by interleukin-6 (IL-6) [13]. Indeed, the activation of IL-6/STAT3 is considered a “bona fide” tumor promoter in obesity [14] and fatty liver-associated inflammation [15].

Interleukin-17 (IL-17) is an essential pro-inflammatory cytokine, which consists of six family ligands, [16]. Previous studies have reported that IL-17 plays an important role in various chronic diseases, including [17, 18] inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis [19]. Recently, evidence has indicated that IL-17 is related to many human cancers, including gastric cancer, breast cancer, and ovarian cancer [20-22]. IL-17 may contribute to HBV-associated liver diseases, and it can stimulate HCC cell migration and invasion [23]. Additionally, Gu et al. found that IL-17 expression was significantly associated with the phosphorylation of STAT3 [24]. Although the correlation between IL-17 and the risk of postoperative recurrence and poor survival of HCC have been verified in previous studies, the underlying mechanisms of IL-17 in modulating HCC cell growth remain elusive [24].

In this study, IL-17 deficient mouse models are used and various experiments are performed to investigate the role of IL-17 in hepatic carcinogenesis induced by HBV. In addition, the interaction between IL-17 and the IL-6/STAT3 signaling pathway is discussed. Through these experiments, our research objective is to identify an alternative target for treating HCC.

Materials and Methods

Cell lines

The cell lines used in the experiment were HepG2 (HBV⁺ HBsAg⁺, human hepatocellular carcinoma cells, purchased from the Institute of Biochemistry and Cell Biology in Shanghai), which were cultured in Dulbecco's modified eagle medium (DMEM). The DMEM in our experiment contained 100 µg/mL streptomycin (HyClone, South Logan, UT, USA), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 250 µg/mL G418 (Sigma, St. Louis, MO, USA). All cells were incubated at 37°C in 5% CO₂. The culture solution was replaced by new media every 2 days. Recombinant human IL-17 (R&D Systems, Minneapolis, MN, USA), an IL-6 neutralizing monoclonal antibody (IL-6 mAb, R&D Systems) and STAT3 inhibitor S31-201 (Selleck, Houston, TX, USA) were applied as indicated.

Cell Counting kit-8 (CCK-8) Test

Cells were seeded onto 96-well plates (2 × 10³ cells per well) and cultured for 1 to 3 days in medium supplemented with recombinant human IL-17 (0, 5, 50 100, 250 and 500 ng/mL). After IL-17 treatment, 10 µL of CCK-8 (Zomanbio, Beijing, China) was added to each well, which was followed by incubation for 3 h. The absorbance at 450 nm for each well was read on a spectrophotometer.

Transwell Assay

The HepG2 cells (3 × 10⁵/mL) treated with recombinant human IL-17, STAT3 inhibitor S31-201 or IL-6 mAb were cultured for 24 h were suspended in DMEM without serum and then added into the upper

chamber of a 6.5-mm Transwell, and the lower chamber was filled with 600 μ L DMEM containing 10% FBS. After incubation for 24 h at 37°C, the transmembrane cells were stained with 0.1% crystal violet. Five point fields were randomly selected to count the numbers of stained cells by microscopy (Olympus, Tokyo, Japan).

Western Blot

HepG2 cells (1×10^5) treated with recombinant human IL-17, STAT3 inhibitor S31-201 or IL-6 mAb were cultured for 24 h. Total protein was extracted from the cells and samples, and the protein concentration was determined by the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Waltham, MA, USA). Equal levels of protein were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred to nitrocellulose membranes; then, the membranes were blocked with 5% nonfat milk for 2 h at room temperature and incubated overnight at 4°C with primary antibodies for STAT3 (ab68153, Abcam, UK, 1:900), p-STAT3 (E121-31, Abcam, 1:1200) and β -actin (ab8227, Abcam, 1:700); β -actin was used for normalization. Then, incubation with secondary antibodies (Beyotime Biotechnology, Shanghai, China) for another 2 h was followed by washing with phosphate buffer (PBS). The signals were detected using the enhanced chemiluminescence substrate kit (Thermo Fisher Scientific). Protein bands were semi-quantified by ImageJ software.

RT-qPCR

Total RNA extraction and reverse transcription were performed as previously described [25]. For frozen tissues, Trizol reagent (Beyotime Biotechnology, China) was used to dissolve the tissues after they were processed. Total RNA was reverse transcribed into cDNA according to the manufacturer's protocol for the RT Kit (#K1622, Fermentas, MA, USA). RT-qPCR was performed using a PCR kit (C10572-014, Invitrogen, Carlsbad, CA, USA) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All primers were synthesized by Shanghai GenePharma Co., Ltd., and the sequences are shown in Table 1. GAPDH was used as an internal control to normalize the IL-6 expression. Differential expression of IL-6 was calculated using the $2^{-\Delta\Delta CT}$ method.

Enzyme linked immunosorbent assay (ELISA) analysis

HepG2 cells (1×10^5) transfected with recombinant human IL-17 were incubated and cultured for 24 h; then, culture supernatants were collected. The protease inhibitor was added into the liver tumor and then tissues were homogenized. After 30 min of 12000 r/min centrifugation, total protein was extracted from the supernatant. The levels of IL-6, MCP-1, CCL5 and VEGF were determined using ELISA kits (R&D Systems) according to the manufacturer's instructions and then quantified by a microplate reader (450 nm).

Immunofluorescence Microscopy

HepG2 cells were collected by trypsin and seeded in glass slides (Thermo Scientific) at a density of 2.5×10^3 . After cultivation for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and then incubated with primary antibodies for STAT3 (Abcam, 1:50) and p-STAT3 (Abcam, 1:50) overnight at 4°C, which was followed by incubation with secondary Alexa fluor 488-labeled (green) anti-IgG or Alexa fluor 555 labeled (red) anti-IgG (Invitrogen). DAPI was used to stain the nuclei. Confocal fluorescence microscopy was used to observe and photograph fluorescent sections.

Heterotopic xenograft liver tumor models

We selected healthy and specific pathogen-free BALB/C mice (5-week-old, female, average body weight of 25 g, purchased from Shanghai Silaike Experimental Animal Co., Ltd.) for the control group. IL-17 KO mice (5-week-old, female, average weight of 26 g) were purchased from Tokyo University and were fed in the Shanghai Southern Research Center. All mice in the group were water and food ad libitum, and they were housed in 12-h light/dark cycles until the date of the experiment. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) management protocol.

Table 1. Primer sequences of GAPDH and IL-6 for implementation of RT-PCR. GAPDH: phosphoglycerate dehydrogenase and RT-PCR: real time-polymerase chain reaction

Gene	Primer sequence
GAPDH	Sense 5'-TGGTATCGTGGAAGGACTCAT-3'
	Antisense 5'-GTGGGTGTCGCTGTTGAAGTC-3'
IL-6	Sense 5'-GCCACTCACCTCTTCAGAACGA-3'
	Antisense 5'-CTGGCTTGTTCTCTCACTACTCT-3'

HepG2 cells (1×10^7 cells in 300 μ L PBS) were subcutaneously injected into the dorsal left flank of 4-week-old male Balb/c nude mice (control and IL-17 KO groups; 5 mice in each group). When the tumor reached approximately 100 mm², the tumor volume was measured every five days with a microcaliper. Tumor volumes (mm³) were estimated by measuring the longest and shortest diameters of the tumors and calculated as previously described. Twenty-five days later, the mice were sacrificed and the tumor weights in the liver were determined.

Orthotopic xenograft liver tumor models

HepG2 cell lines were stably transfected using the pGL3-control vector (Promega, Madison, WI, USA) and pSV2Neo (ATCC, USA), as previously described [26]. The cells were treated with 10 μ g of pGL3-control and 1 μ g of pSV2Neo vectors using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) and then selected with geneticin (400 μ g/mL). Stable HepG2 cells expressing Luc were isolated and the clone with the highest Luc expression level (measured by its bioluminescence) was sorted with luciferin (Xenogen, CA, USA) and an *in vivo* imaging system (Xenogen).

HepG2-Luc cells were dispersed in suspension using pancreatin and the suspension was centrifuged (1000 rpm, 5 min) and mixed with PBS before it was pumped into 1-mL sterile syringes. A skin incision was made under the breastbone along the left costal margin. From the incision, muscles and peritoneal layers were bluntly separated to expose part of the left liver. Then, we injected 50 μ L of cell suspension (1×10^7 tumor cells in 0.3 mL of PBS) into the left lobe of the liver (both control group and IL-17 KO groups; 3 mice in each group). After regaining consciousness, mice were fed sterilely.

After aqueous solution of luciferin (150 mg/kg intraperitoneally) was injected 10 min prior to imaging, mice were anesthetized and placed into a light-tight chamber of the CCD camera system (Xenogen). The photons emitted from the luciferase-expressing cells within the animal were quantified for 5 min using Living Image software (Xenogen) as an overlay.

After 10 days of tumor growth, mice were euthanized and their liver tissues were fixed with 10% formalin. The formalin-fixed tissues were embedded in paraffin, cut into 5- μ m sections, placed onto glass slides, and used to count the total number of tumors with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO, USA) for histological examination. The concentrations of IL-6, MCP-1, CCL5 and VEGF in the liver were detected with an ELISA kit according to the manufacturer's instructions.

Statistical Analysis

SPSS19.0 was used to analyze the results, which are expressed as the median (percent or mean $\pm$ standard deviation (SD)). When two groups were compared, the unpaired Student's t-test was used. When multiple groups were compared, multivariate analysis of variance (ANOVA) was used. A Mann-Whitney U test was used to calculate statistical significance. $P < 0.05$ was considered indicative of a significant difference.

Results

IL-17 enhances the proliferation and migration of HepG2 cells

The CCK-8 assay was used to detect the proliferation of HepG2 cells after treatment with different concentrations of IL-17. As shown in Fig. 1A, IL-17 accelerated the cell proliferation in a concentration-dependent manner with a remarkable growth rate in the IL-17 concentration of 100 ng/mL. Hence, we selected 100 ng/mL IL-17 in the subsequent experiments. The Transwell assay was used to measure the migration of HepG2 cells. As shown in Fig. 1B, exogenous IL-17 (100 ng/mL treatment for 24 h) remarkably enhanced the migration of HepG2 cells compared to the Control group ($P < 0.05$). The results indicated that IL-17 can promote the proliferation and migration of HepG2 cells.

IL-17 promotes STAT3 phosphorylation

It is well known that the STAT3 pathway plays a significant role in maintaining hepatocellular carcinoma [27]. Therefore, we predicted that IL-17 might maintain the self-renewal of HepG2 cells through the STAT3 pathway. To verify this hypothesis, we conducted Western Blot and immunofluorescence analyses. As shown in Fig. 2A, IL-17 enhanced the

level of phosphorylation of STAT3, while the total STAT3 level was unchanged. Next, we used STAT3 inhibitor, S31-201, or combined utilization of IL-17 (100 ng/mL) and S31-201 to treat HepG2 cells and found that S31-201 could efficiently block the phosphorylation of STAT3, even in the presence of IL-17. Therefore, exogenous IL-17 could not reverse the inhibition effects of S31-201. Immunofluorescence results confirmed the Western Blot results (Fig. 2B). The cytokines associated with the STAT3 pathway were measured using an ELISA. The ELISA results shown in Fig. 2C revealed IL-17 treatment increased the expression levels of MCP-1,

Fig. 1. IL-17 enhances the proliferation and migration of HepG2 cells. (A) Effects of IL-17 on the proliferation of HepG2 cells were detected using the CCK-8 assay. (B) Effects of IL-17 on the migration of HepG2 cells were detected by a Transwell assay (magnification, $\times 100$). Data are presented as the mean $\pm$ SD for three independent experiments. ** $P < 0.05$ compared to Control.

Fig. 2. IL-17 activates the phosphorylation of STAT3. (A) Western Blot analysis of p-STAT3 and STAT3 expression in HepG2 cells after treatment with IL-17 and S31-201. (B) Quantitation of p-STAT3 and STAT3 protein expression using immunofluorescence microscopy in HepG2 cells with IL-17 and S31-201 treatment; p-STAT3 was stained green, STAT3 was stained red and cell nuclei were stained with DAPI. (C) ELISA performed to detect the level of cytokines related to the STAT3 pathway, including MCP-1, CCL5 and VEGF. Data are presented as the mean $\pm$ SD for three independent experiments. ** $P < 0.05$ compared to Control. Scale bar = 50 μ m.

Fig. 3. IL-17 activates the STAT3 pathway through IL-6 (A) The effects of IL-17 on the mRNA expression of IL-6 in HepG2 cells were detected by the RT-qPCR assay. (B) Western Blot analysis of p-STAT3 and STAT3 expression in HepG2 cells after treatment with IL-17 and IL-6 mAb. (C) ELISA performed to detect the levels of IL-6, MCP-1, CCL5 and VEGF. Data are presented as the mean $\pm$ SD for three independent experiments. ** $P < 0.05$ compared to Control.

CCL-5 and VEGF compared with the control group ($P < 0.05$), while the inhibitor of STAT3 reduced the levels of MCP-1, CCL-5 and VEGF ($P < 0.05$). Treatment of IL-17 could efficiently attenuate the inhibition of S31-201 on the indicated cytokines, which further verified the activation of STAT3 in the IL-17 treatment group.

IL-17 regulated the STAT3 pathway through IL-6

To investigate whether IL-17 regulated the STAT3 pathway through producing IL-6, we detected the expression of IL-6 in the presence or absence of IL-17. The expression of IL-6 mRNA was measured by RT-qPCR (Fig. 3A), and it was shown that IL-17 promotes the expression of IL-6 ($P < 0.05$).

As before, we showed that IL-17 promotes the expression of p-STAT3. To determine whether the IL-17-mediated STAT3 pathway depended on IL-6, we used IL-6 neutralizing mAb (IL-6 mAb, 20 ng/mL), or combined utilization of IL-17 (100 ng/mL) and IL-6 mAb (20 ng/mL) to treat HepG2 cells. We found that IL-6 mAb could efficiently block the phosphorylation of STAT3 in the absence of IL-17. However, exogenous IL-17 could neutralize the inhibition effects of IL-6 mAb (Fig. 3B). The ELISA was also performed and the MCP-1, CCL5, VEGF and IL-6 levels were measured. As shown in Fig. 3C, although IL-6 mAb significantly suppressed the levels of IL-6, MCP-1, CCL5 and VEGF compared with the control group ($P < 0.05$), IL-17 could efficiently relieve the downregulation of IL-6, MCP-1, CCL5 and VEGF, which were tightly related to the STAT3 pathway. Taken together, IL-17 activation of the STAT3 pathway depended on IL-6.

IL-17 promotes the proliferation and migration of HepG2 cells via the IL-6/STAT3 pathway

The CCK-8 and Transwell assays were used to detect the proliferation and migration of HepG2 cells. As shown in Fig. 4A and 4B, IL-17 significantly accelerated cell proliferation and migration, while S31-201 or IL-6 mAb remarkably attenuated cell proliferation and migration compared to Control cells ($P < 0.05$). Meanwhile, IL-6 mAb+IL-17 co-treatment had little effect on cell proliferation and migration compared to Control cells ($P > 0.05$), but S31-201+IL-17 co-treatment significantly attenuated cell proliferation and migration ($P < 0.05$). The results indicated that IL-17 can promote the proliferation and migration of HepG2 cells, but STAT3 inhibitor S31-201 or IL-6 mAb significantly reversed the promoting effects

Fig. 4. IL-17 enhances the proliferation and migration of HepG2 cells via the IL-6/STAT3 pathway. (A) Effects of the IL-6/STAT3 pathway on the proliferation of HepG2 cells were detected using CCK-8 assay. (B) Effects of the IL-6/STAT3 pathway on the migration of HepG2 cells were detected by a Transwell assay (magnification, $\times 100$). Data are presented as the mean $\pm$ SD for three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared to Control.

Fig. 5. IL-17 knockout suppressed the proliferation of tumor in heterotopic tumor models. (A) Tumor volumes were measured after stable formation. IL-17 knockout suppressed tumor growth *in vivo*. B: Samples of the xenograft models at 25 days. C: Mice were sacrificed 25 days later, and tumors were extracted and weighed. IL-knockout decreased the tumor weight *in vivo*. Data are presented as the mean $\pm$ SD. ** $P < 0.05$ compared to wild type mice.

of proliferation and migration from IL-17 stimulation in HepG2 cells ($P < 0.05$). According to those results, we verified that IL-17 activation of the STAT3 pathway depended on IL-6; therefore, IL-6/STAT3 activation was suggested to be responsible for the promoting effects of IL-17 in the proliferation and migration of HepG2 cells.

Effects of the IL-17/IL-6/STAT3 signaling pathway on the tumor proliferation in mice

To investigate whether IL-17 enhances HepG2 cell proliferation *in vivo*, we injected HepG2 cells or luciferase-labeled HepG2 cells into nude mice (wild type and IL-17 knockout mice). HepG2 cells were subcutaneously injected into the dorsal left flank of Balb/c mice to construct heterotopic xenograft models. After the tumor was stably 100 mm³ in size, the tumor growth was measured with a microcaliper, as shown in Fig. 5A. The tumor volume in wild type mice increased more quickly than in IL-17 knockout mice ($P < 0.05$). Mice were sacrificed at 25 days. The tumor samples are shown in Fig. 5B, and the tumor weights are compared in Fig. 5C. The tumor volume and weight were higher in wild type mice than IL-17 knockout mice ($P < 0.05$). That is, IL-17 gene knockout attenuated tumor growth *in vivo*.

HepG2-Luc cells were injected into the left lobe of the liver to construct orthotopic xenograft models. Living images of mice at 10 day post injection are shown in Fig. 6A. The sum of the tumor tissues from liver sections in wild type and IL-17 knockout mice were calculated. As shown in Fig. 6B, IL-17 significantly attenuated tumorigenesis *in vivo*. Liver histological examination was performed, and the density and size of liver tumor tissues of wild mice were higher than for IL-17 knockout mice (Fig. 6C). Total protein was extracted from mice livers; then, the expression of STAT3 and p-STAT3 were quantitatively tested by Western Blot. As shown in Fig. 6D, decreased phosphorylation of STAT3 was evident, while the total STAT3 was unchanged. The concentrations of IL-6, MCP-1, CCL5 and VEGF were detected by ELISA. The results shown in Fig. 6E revealed that the concentrations of IL-6, MCP-1, CCL5 and VEGF were significantly downregulated in IL-17 knockout mice compared with the concentrations in wild type mice ($P < 0.05$). The lower expression of these cytokines in the IL-17 knockout group also verified the decreasing activation of the STAT3 pathway by IL-17 knockout *in vivo*.

Fig. 6. IL-17 knockout suppressed tumor proliferation in orthotopic tumor models. (A) Samples of the xenograft models at 10 days after injection. (B) The tumor numbers were calculated, and IL-17 attenuated tumorigenesis *in vivo*. (C) Effects of IL-17 knockout on the density of liver tumor were detected by liver histological examination. (D) Effects of IL-17 knockout on the expression of p-STAT3 *in vivo* were detected by Western Blot. (E) Effects of IL-17 knockout on the expression levels of IL-6, MCP-1, CLL5 and VEGF *in vivo* were detected by ELISA. Data are presented as the mean $\pm$ SD. ** $P < 0.05$ compared to wild type mice.

Discussion

Hepatocellular carcinoma (HCC), the fifth most common tumor, is the third most common cause of death related to cancer worldwide [28]. Chronic inflammatory states often occur after the development of HCC because of cirrhosis and chronic hepatitis stimulated by either hepatitis B/C virus or non-viral-correlated factors, including alcohol and obesity [29]. Convincing evidence has shown that the immune microenvironment around the liver in HCC could play a significant role in the proliferation, migration, and survival of cancer cells [30].

IL-17 is a primary cell factor secreted by Th17 cells, which plays a crucial role in promoting tumor growth [31]. Although the exact mechanism was not well clarified, many publications reported that IL-17 could exert a notable effect on promoting the growth of cervical tumor, non-small cell carcinoma, breast cancer, colon cancer and papilloma [32-35]. Zhang et al. demonstrated that IL-17+ T cells were discovered in large numbers within HCC and were associated with poor survival and improved recurrence, suggesting that Th17 cells and IL-17 could facilitate HCC progression [36]. However, the direct effect and potential mechanism of IL-17 in regulating human HCC cell growth remain incompletely defined. In the present study, we found that IL-17 could facilitate the proliferation and migration of HCC *in vitro* and in IL-17^{-/-} mice, which was similar to the finding by Zhang et al. [36].

STAT3 is a cancer gene that is activated by IL-17 in tumor or stromal cells, which directly enhances tumor growth and metastasis [37]. IL-17 was reported to modulate signaling through distinct pathways, such as the MAPK, NF-κB, and STAT3 pathways, in various inflammatory and tumor cells [38]. In our study, we found that IL-17 could increase the phosphorylation of STAT3 and promote STAT3 activation *in vitro* and in IL-17^{-/-} mice, which was consistent with findings of Wang et al. and Gu et al. [24, 39].

IL-6 is a STAT3 activating factor that is increased in numerous cancers [40]. IL-6 and other members of the IL-6 family, which can result in cancer-promoting inflammation by inducing the JAK-STAT3 pathway, have been widely reported [41]. Additionally, IL-6 plays an important role in promoting the phosphorylation of both STAT3 and Jak2 [42]. Activation of the STAT3 by phosphorylation in response to IL6 turns on the transcriptions of many downstream genes involved in normal hepatic development [43]. The IL-6-induced STAT3 signaling pathway in tumor cells enhances proliferation, proangiogenic, and antiapoptotic genes. It also leads to inhibition of several pro-inflammatory genes [44]. Therefore, we investigated whether IL-6 caused IL-17-driven IL-6 induction of STAT3 activation in HCC. Then, we found that IL-17 could increase the expression level of IL-6. Furthermore, anti-IL-6 antibodies abrogated IL-17-induced STAT3 activation. These results elucidated that IL-17 could activate STAT3 by promoting the expression of IL-6 in HCC, which was consistent with the findings of Wang et al. and Gu et al. [24, 39].

Recently, substantial research found that IL-17 could positively induce elevation of IL-6 expression and activation of STAT3 in fibroblasts and inflammatory cells surrounding autoimmune diseases [45]. Wang et al. suggested that IL-17 exerted a direct effect on facilitating the growth of tumor cells via activating the IL-6/STAT3 pathway by improving the expression of IL-6 and a signal transducer in murine B16 melanoma and MB49 bladder carcinoma [39]. Gu et al. suggested that IL-17 exerted a direct effect on promoting HCC growth through the inducing the IL-6/JAK2/STAT3 by activating the AKT pathway [24]. In agreement with the above findings, we clarified the related molecular mechanism of IL-17 in the proliferation and migration of HCC cells in the present study, suggesting that IL-17 may play a significant role in facilitating tumor growth in HCC by activating the IL-17/IL-6/STAT3 pathway *in vitro* and in IL-17^{-/-} mice.

Excluding the above findings about the effect of IL-17 in facilitating tumor growth, our findings contrasted with those in other reports that IL-17 could have an antitumor effect for various tumors. For example, Kortylewski et al. found that tumor growth of MC38 sarcoma

was promoted in IL-17^{-/-} mice [46]. These findings demonstrated that the action of IL-17 in tumor highly depended on the cell type and environment background in modulating the proliferation and metastasis of tumor cells, supporting that the role of IL-17 in HCC requires specific investigation. Further research is needed to discuss the direct effect and mechanism of IL-17 on the biological behavior of tumor cells as well as to clarify why IL-17 has contrasting roles in regulating tumor growth in distinct tumor systems.

Taken together, we have confirmed that IL-17 could facilitate tumor growth. Moreover, our study demonstrated that the tumor-promoting effect mediated by IL-17 might be correlated with a direct action on tumor cells through inducing IL-6, which could activate STAT3 in HCC. Our results indicated that IL-17, STAT3 and IL-6 might act as molecular targets for the diagnosis, treatment and prognosis of hepatitis B virus-related HCC.

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Disclosure Statement

None.

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