

B7-H4 facilitates proliferation of esophageal squamous cell carcinoma cells through promoting interleukin-6/signal transducer and activator of transcription 3 pathway activation

Xinran Chen,¹ Ling Wang,² Wei Wang,^{1,3} Lianmei Zhao¹ and Baoen Shan^{1,2}

¹Research Center, Fourth Hospital of Hebei Medical University, Shijiazhuang; ²Hebei Cancer Research Institute, Fourth Hospital of Hebei Medical University, Shijiazhuang; ³Clinical Laboratory, Hebei General Hospital, Shijiazhuang, China

Key words

Apoptosis, B7-H4, cell proliferation, esophageal squamous cell carcinoma, IL-6/STAT3 signal pathway

Correspondence

Baoen Shan, Fourth Hospital of Hebei Medical University, Tianshan Street No. 169, Hightech Development Zone, Shijiazhuang City, Hebei Province 050035, China.
Tel: +86-15030185856; Fax: +86-0311-66696289;
E-mail: hbhshan@163.com

Funding Information

National Natural Science Foundation of China (81173611); Major Research Project of Hebei Province (zd2013045); Hebei Education Department.

Received December 14, 2015; Revised April 5, 2016;
Accepted April 14, 2016

Cancer Sci 107 (2016) 944–954

doi: 10.1111/cas.12949

B7-H4, one of the costimulatory molecules of the B7 family, has been found to be widely expressed in many kinds of tumor tissues and to play an important part in tumor progression and poor prognosis. However, the role of B7-H4 in esophageal squamous cell carcinoma (ESCC) cells has not been elucidated. In this study, we found that, compared with normal esophageal tissue, B7-H4 was highly expressed in three ESCC cell lines, Eca109, TE1, and TE13. B7-H4 silenced cells suppressed cellular proliferation and colony formation. Additionally, compared with control cells, B7-H4 silenced cells showed higher apoptosis rates, Bcl-2 and Survivin upregulation, and BAX downregulation. Further study revealed that B7-H4 silenced cells also showed reduction in interleukin-6 (IL-6) secretion, signal transducer and activator of transcription 3 (STAT3) activation, and p-STAT3 translocation from cytoplasm to nucleus. Moreover, B7-H4 depletion inhibited the IL-6 secretion of control cells but not JAK2/STAT3 inhibitor FLLL32-treated cells. Interleukin-6 receptor antagonist tocilizumab did not block the p-JAK2 or p-STAT3 downregulation induced by B7-H4 silence. It was suggested that B7-H4 silence suppressed IL-6 secretion through JAK2/STAT3 inactivation. Furthermore, cell proliferation and colony formation were downregulated by tocilizumab in control cells but not in B7-H4 silenced cells, indicating that IL-6 upregulation induced by B7-H4 was necessary for cell growth. On the other hand, B7-H4 expression was downregulated by tocilizumab. In all, our study provided the first evidence that B7-H4 facilitated ESCC cell proliferation through promoting IL-6/STAT3 positive loopback pathway activation.

Esophageal cancer is the eighth most common cancer with the sixth highest mortality worldwide.⁽¹⁾ According to the pathological types, esophageal cancer can be classified into squamous cell cancer and adenocarcinoma.⁽²⁾ Although the incidence of adenocarcinoma has been increased rapidly in North America during the past 30 years,⁽³⁾ esophageal squamous cell cancer (ESCC) is still the most common threat in Asia, southern and eastern Africa, as well as northern France.⁽⁴⁾ The disease is especially prevalent in Asia, where ESCC accounts for approximately 90% of esophageal cancer.⁽⁵⁾ Unfortunately, the 5-year survival rate of ESCC is only around 20%⁽⁶⁾ because most cases are diagnosed in the advanced stage when no effective treatment strategies are available.⁽⁷⁾ Therefore, there is an urgent need to investigate the pathogenesis of ESCC and explore new therapeutic targets.

The B7 family, consisting of costimulatory molecules, plays an irreplaceable role in the regulation of immune response and immune evasion of tumor. In addition to the traditional B7-1 and B7-2 family members, other B7 family members have been discovered, including B7-H1,⁽⁸⁾ B7-H2,⁽⁹⁾ B7-H3,⁽¹⁰⁾ B7-

H4,⁽¹¹⁾ B7-DC,⁽¹²⁾ and B7-H6.⁽¹³⁾ In 2003, B7-H4 was identified by three different laboratories simultaneously and was designated three different names, B7-H4,⁽¹¹⁾ B7s1,⁽¹⁴⁾ and B7x.⁽¹⁵⁾ Previous studies observed that B7-H4 mRNA is broadly expressed in human peripheral tissues whereas B7-H4 protein expression was confined only to some tumor tissues, such as lung,⁽¹⁶⁾ ovarian,⁽¹⁷⁾ prostate,⁽¹⁸⁾ melanoma,⁽¹⁹⁾ stomach,⁽²⁰⁾ breast,⁽²¹⁾ kidney,⁽²²⁾ and esophagus.⁽²³⁾ It has been proved that B7-H4 plays an important role in regulating adaptive immune response by inhibiting the proliferation, activation, and cytokine production of T cells, and host innate immune response by suppressing growth of neutrophil progenitors.⁽²⁴⁾ In addition, Cheng *et al.*⁽²⁵⁾ found that B7-H4-deficient mice showed lower ovarian tumorigenicity and Qian *et al.*⁽²⁶⁾ reported that B7-H4 knocked down pancreatic cells showed lower proliferation and higher apoptosis. It was suggested that B7-H4, as an important tumor marker, was able to promote cancer cell proliferation and tumorigenesis independently of lymphocytes.

Signal transducers and activators of transcription (STATs) are cytoplasmic transcription factors that play a key role in cell

fate. STAT3, a protein family comprised of seven members, conveys signals from the cell surface to the nucleus through activation by cytokines and growth factors.⁽²⁷⁾ Interleukin-6 (IL-6), activator and target molecule of STAT3, plays an important part in diverse biological functions, including cell differentiation, proliferation, development, apoptosis, and inflammation.⁽²⁸⁾ Interestingly, it has been identified that IL-6 successfully stimulated B7-H4 protein expression on macrophages, which suppressed T cell proliferation *in vitro*.⁽²⁹⁾ Furthermore, macrophages also stimulated B7-H4 expression in an autocrine manner through IL-6.⁽³⁰⁾

Although Chen and coworkers reported that B7-H4 is widely expressed in ESCC tissues and positively correlated with tumor progression and poor prognosis,⁽²³⁾ the pertinent mechanism was not clear. Additionally, we wondered whether there is a relationship between B7-H4 expression and the IL-6/STAT3 pathway. So, in this paper, we studied the effect of B7-H4 on ESCC cell proliferation and the correlation between B7-H4 and IL-6/STAT3 signal pathway activation.

Materials and Methods

Cell lines. Human ESCC cell lines, Eca109, TE1, and TE13, were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Sijiqing, Shanghai, China). The cells were maintained in an incubator with 5% CO₂/95% air at 37°C.

B7-H4 transfection. B7-H4 expression was knocked down by B7-H4 shRNA (Origene Technologies, Rockville, MD, USA). The control shRNA contained a non-effective shRNA cassette against GFP. The set sequence of the B7-H4 shRNA was 5'-GTGGACTATAATGCCAGCTCAGAGACCTT-3'. For the transfection process, approximately 5×10^5 cells were seeded in 6-well plates. After 24 h of culture, 2 µg transfect DNA and 4 µL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in RPMI-1640 medium without FBS was added into each well to incubate for 6 h. Then the medium was exchanged by RPMI-1640 with 10% FBS, followed by 48 h of incubation. The cells were then harvested for analysis.

Cell proliferation. Harvested cells were seeded in 96-well plates at 4000 cells per well. After 0, 24, 48, and 72 h incubation, 20 µL [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega, Madison, WI, USA) was added to each well and incubated for 3 h in an incubator at 37°C. Finally, the absorbance at 490 nm was measured.

Colony formation. Harvested cells were calculated and 800 cells were seeded per well. After culturing for 12 days, cells were fixed with 4% formaldehyde for 30 min and stained with 1% crystal violet for 20 min. After being washed with PBS three times, images of cell colonies were captured and analyzed.

Flow cytometry for cell apoptosis. Cell apoptosis was assayed with an annexin V- phycoerythrin/7-amino-actinomycin D apoptosis detection kit (BD, San Jose, CA, USA). In brief, 1×10^6 cells for every sample were harvested, then washed with PBS and incubated with 5 µL annexin V- phycoerythrin, and 5 µL 7-amino-actinomycin D for 15 min at room temperature. Finally, cells were analyzed with BD flow cytometry to determine apoptosis profiles.

Quantitative real-time PCR. Total RNA was extracted from cell samples using a TRI reagent RNA isolation reagent

(Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Total RNA (5 µg) for each sample was reverse transcribed to first-strand DNA (cDNA) with a reverse transcription system (Promega). The PCR was carried out in a total volume of 20 µL reaction mixture containing cDNA product (2 µg), specific primers (0.2 µmol/L), SYBR-Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and nuclease-free water. The sequences of primers were as follows: B7-H4, 5'-TATTAGCAGCCGCTCTGTGC-3' (sense) and 5'-TCAGGATTCCATCCTCCCA-3' (antisense); IL-6, 5'-CCATCCAGTTGCCTTCTTGG-3' (sense) and 5'-CTCCTCTCCGACTTGTGAA-3' (antisense); B-cell lymphoma 2 (Bcl-2), 5'-CTGAAGACCCTCAGGATGCG-3' (sense) and 5'-TGGCCTTGATGACACCTTGG-3' (antisense); Bcl-2 associated X protein (BAX), 5'-GCTGAACCAAGGAGACGGAA-3' (sense) and 5'-AGAAGTTGGCATGGTAGCCC-3' (antisense); Survivin, 5'-GCTGAACCAAGGAGACGGAA-3' (sense) and 5'-AGAAGTTGGCATGGTAGCCC-3' (antisense); STAT3, 5'-AGCCTCAGGAAGCGGAAAAG-3' (sense) and 5'-TGAGTTCA GTCAGCCGCTTG-3' (antisense); and GAPDH, 5'-GTCAA CCGATTTGGTTCGATTG-3' (sense) and 5'-CATGGGTGGAATCATATTGGAA-3' (antisense). The amounts of target transcripts were calculated and normalized with housekeeping gene *GAPDH* in the samples. The PCR was programmed as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 1 min. Differences in the expression levels of genes were determined by calculating the fold change in expression ($2^{-\Delta\Delta CT}$).

Western blot analysis. Total proteins were extracted with a Total Extraction Kit (Solarbio, Beijing, China). Cytoplasmic and nuclear proteins were extracted with a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime, Shanghai, China). Concentrations of proteins were detected by a Bicinchoninic Acid kit (Sigma-Aldrich).

The Western blot analysis was carried out as described previously.⁽³¹⁾ The transfer times were: 30 min for GAPDH, TATA-binding protein (TBP), Bcl-2, BAX, and Survivin; 1 h for B7-H4, STAT3, and p-STAT3; and 2 h for JAK2 and p-JAK2. The antibodies included: rabbit anti-human mAbs against Bcl-2, BAX, Survivin, STAT3, p-STAT3, JAK2, p-JAK2 (Cell Signaling Technology, Beverly MA, USA), B7-H4 (Genetex, Irvine, CA, USA), and rabbit anti-human polyclonal antibody against GAPDH (Rockland, Philadelphia, PA, USA) and TBP (Proteintech, Chicago, IL, USA). After incubation with the above primary antibodies overnight at 4°C, the membranes were incubated with fluorescent rabbit secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 37°C. The immunoreactive bands were determined by image scanning on the Odyssey fluorescence scanner (LI-COR Biosciences, Lincoln, NE, USA) and analyzed with the image software.

Immune fluorescence staining. Cells harvested were fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized in 0.15% Triton X-100 for 10 min, blocked in 3% BSA at room temperature for 30 min and incubated with rabbit to human STAT3 or p-STAT3 mAb at 4°C overnight. The cells were then stained by Alexa Fluor 594 conjugated goat anti-rabbit antibody (Proteintech) at 37°C for 1 h, followed by DAPI staining of the nucleus (Beyotime). The fluorescence was observed and analyzed with a fluorescence microscope at high magnification ($\times 400$).

Silencing of STAT3 by FLLL32 and IL-6 detection by ELISA. Cells were treated with control shRNA or B7-H4 shRNA for 6 h, then cultured in 10% FBS medium with or without JAK2/STAT3 inhibitor, 5 µM FLLL32 (Selleck Chemicals, Houston,

TX, USA), for 48 h. Consequently, the culture supernatant was collected for IL-6 detection following ELISA kit instructions (Lianke, Shanghai, China).

Effect of tocilizumab on B7-H4 activating JAK2/STAT3. Cells were treated with control shRNA or B7-H4 shRNA for 6 h, then cultured in 10% FBS medium with or without IL-6 receptor antagonist, 200 ng/mL tocilizumab (Roche, London, UK), for 48 h. The cells were harvested then Western blot assay was used to detect the protein expression of p-JAK2, total JAK2, p-STAT3, and total STAT3.

Effect of tocilizumab on ESCC growth and B7-H4 expression. Cells pretreated with control shRNA or B7-H4 shRNA were harvested and subjected to MTS and colony formation assays following the process above. The cells were cultured in normal medium, with or without 200 ng/mL tocilizumab.

To determine the effect of IL-6 on B7-H4 expression in ESCC cells, 200 ng/mL tocilizumab was added to Eca109, TE1, and TE13 cells. After 48 h of treatment, cells were harvested and Western blot assay was used to detect the protein expression of B7-H4.

Effect of tocilizumab on Eca109 tumorigenesis in BALB/c mice. Twelve BALB/c mice (male, 5–6 weeks old, obtained from Beijing Weitonglihua Experimental Animal Co., Beijing, China) were raised in a specific pathogen-free animal laboratory. Human Eca109 cells, 5×10^6 in 0.2 mL PBS, were s.c. injected into the right front leg of every mouse. The 12 mice were divided into two groups, control and tocilizumab (six mice per group). Tocilizumab at 20 mg/kg was injected i.p. at 6, 9, 12, 15, and 18 days after the cells were injected. Tumor volumes were measured once every 3 days. Twenty-one days after cell implantation, the mice were killed and tumors were removed and weighed. Protein extracts from the tumors were isolated to detect B7-H4 protein expression by Western blot analysis. This study was carried out in accordance with the care guidelines of laboratory animals of Hebei Medical University.

Statistical analysis. Results were reported as mean \pm SD. All the experimental data were analyzed by the GraphPad Prism (GraphPad Software, La Jolla, CA, USA) 5.0 statistical software package. Student's *t*-test or one-way ANOVA was used to compare the difference between two or more groups. A *P*-value < 0.05 (two-tailed) was considered statistically significant.

Results

B7-H4 highly expressed in ESCC cells. To evaluate B7-H4 expression in ESCC cells, we chose three cell lines with different conditions of differentiation; Eca109 and TE1 cell lines were highly differentiated whereas TE13 cells were poorly differentiated. Interestingly, we found that, compared with normal esophageal tissue, B7-H4 was highly expressed in all three cell lines at a similar level (Fig. 1a).

B7-H4 silence suppressed ESCC proliferation and colony formation. In order to investigate whether B7-H4 can affect the biological function of ESCC cells, we knocked down the B7-H4 expression by B7-H4 shRNA. Results of Western blot analysis showed that, 48 h after transfection, B7-H4 expression was markedly inhibited in cells pretreated with B7-H4 shRNA compared with cells pretreated with control shRNA (Fig. 1b).

First, we detected the effect of B7-H4 on ESCC proliferation by MTS assay. Cells harvested after transfection showed normal morphology, observed by microscope. Compared with control cells, B7-H4 silenced cells showed significantly lower proliferation rates, particularly at 48 and 72 h after planted

(Fig. 1c). Then, colony formation ability was tested. In this study, we found that knockdown of B7-H4 expression in Eca109, TE1, and TE13 cells strongly reduced the colony numbers ($P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 1d). Together, these data suggested that B7-H4 played an important role in facilitating the ESCC proliferation and colony formation.

B7-H4 silence promoted apoptosis of ESCC cells. Cell apoptosis, also known as programmed cell death, is a basic physiological phenomenon of cells. In general, the excessive proliferation of cancer cells is often accompanied by apoptosis suppression. Thus, we speculated that the promoting effect of B7-H4 to cell proliferation may be related to apoptosis inhibition. In support, we detected its effect on the apoptosis profiles of ESCC cells. Result of flow cytometry (Fig. 2a) indicated that B7-H4 depletion promoted total apoptosis in Eca109, TE1, and TE13 cells ($P < 0.001$, $P < 0.01$, and $P < 0.05$, respectively). Early apoptosis and late apoptosis were also increased in B7-H4 silenced cells to a certain extent. Additionally, we found that, compared with control cells, apoptosis-inhibiting molecules Bcl-2 and Survivin were downregulated, whereas apoptosis-promoting molecule BAX was upregulated in B7-H4 silenced cells at both gene and protein levels detected by qRT-PCR and Western blot assays (Fig. 2b,c). Together, these data indicate that B7-H4 silence promoted the apoptosis of ESCC cells, contributing to the proliferation inhibition of ESCC cells.

B7-H4 depletion repressed IL-6 secretion through JAK2/STAT3 inactivation. During the detection of cell culture supernatant, interestingly, we found that the IL-6 level of B7-H4 silenced cells was significantly lower than that of control cells. Accordingly, we detected its gene expression in ESCC cells. Results of qRT-PCR showed that *IL-6* gene expression in B7-H4 silenced Eca109, TE1, and TE13 cells was strongly dampened compared with that of control cells ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) (Fig. 3a).

It is well known that IL-6 expression is closely linked with STAT3 activation.⁽³²⁾ More precisely, there is a positive feedback loop between IL-6 and STAT3 activation. Additionally, it has been reported that STAT3 activation could regulate apoptosis-relative protein expression, such as Bcl-2 members.⁽³³⁾ Is there any relationship between B7-H4 expression and IL-6/STAT3 activation? We tested STAT3 expression and activation. Results in Figure 3(a,b) show that, compared with control cells, cells pretreated with B7-H4 shRNA showed lower expression of phosphorylation of STAT3 (p-STAT3), the active form of STAT3, although there was no obvious change in total STAT3. It was suggested that B7-H4 silence led to inhibition of STAT3 activation. Furthermore, we investigated the effect of B7-H4 on JAK2, a critical tyrosine kinase of activating STAT3. As shown in Figure 3(c), B7-H4 silence downregulated p-JAK2 expression in Eca109 ($P < 0.01$), TE1 ($P < 0.05$), and TE13 ($P < 0.01$), cells although the total JAK2 expression was not obviously influenced.

Translocation of p-STAT3 from cytoplasm to nucleus and then binding to the target gene is the key step of STAT3 pathway activation in starting the downstream gene expression.⁽³²⁾ Accordingly, we further investigated the location of STAT3 by immune fluorescence detection. STAT3 is mainly located in the nucleus, with little distribution in the cytoplasm. B7-H4 did not obviously affect STAT3 expression in either the nucleus or cytoplasm. Similarly, p-STAT3 also mainly located in the nucleus of Eca109, TE1, and TE13 cells. However, the expression of p-STAT3 in the nucleus was decreased

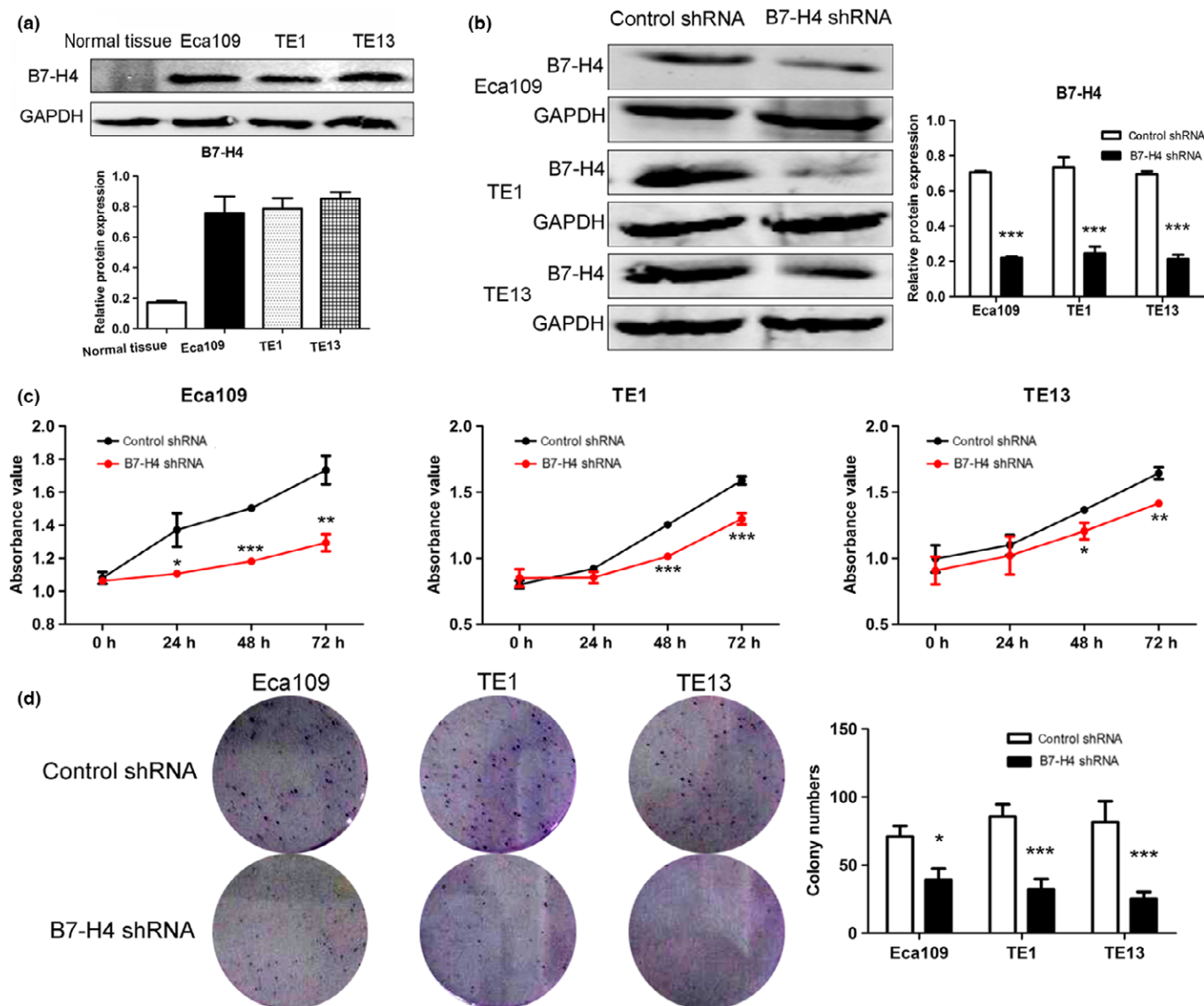


Fig. 1. B7-H4 was highly expressed in Eca109, TE1, and TE13 esophageal squamous cell carcinoma cells and B7-H4 silence inhibited cell proliferation. (a) Compared with normal esophageal tissue, B7-H4 was highly expressed in Eca109, TE1, and TE13 cells analyzed by Western blot. No significant difference in B7-H4 expression was found among these cells (all $P > 0.05$). (b) B7-H4 expression was successfully knocked down by B7-H4 shRNA compared with cells pretreated with control shRNA in Eca109, TE1, and TE13 cells (all $P < 0.001$) detected by Western blot. (c) B7-H4 depletion inhibited ESCC cell proliferation, especially at 48 and 72 h. (d) B7-H4 depletion significantly inhibited cells colony numbers in Eca109, TE1, and TE13 cells ($P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively) by colony formation detection. All data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA.

significantly in cells pretreated with B7-H4 shRNA compared with that of cells pretreated with control shRNA (Fig. 4a). These results were confirmed by Western blot assay. As shown in Figure 4(b), low B7-H4 expression led to lower p-STAT3 expression in the nucleus of Eca109 ($P < 0.01$), TE1 ($P < 0.05$), and TE13 ($P < 0.05$) cells. No significant difference in p-STAT3 expression was observed in the cytoplasm.

In terms of IL-6 secretion, we found that B7-H4 silence markedly downregulated IL-6 secretion in Eca109, TE1, and TE13 cells. In contrast, compared with FLLL32 and control shRNA-treated cells, IL-6 secretion in Eca109, TE1, and TE13 cells pretreated with B7-H4 shRNA and FLLL32 was not significantly affected (Fig. 5a). These data suggested that B7-H4 silence inhibited IL-6 secretion in control cells but not JAK2/STAT3 blocked cells. JAK2/STAT3 activation is necessary for B7-H4 facilitating IL-6 secretion.

As Figure 5(b) shows, B7-H4 depletion inhibited p-JAK2 and p-STAT3 expression of control cells. B7-H4 silence also markedly suppressed p-STAT3 expression in Eca109, TE1, and TE13 cells pretreated with tocilizumab (all $P < 0.05$). Expression of p-JAK2 in TE1 and TE13 cells cultured with tocilizumab was also significantly inhibited by B7-H4 depletion ($P < 0.05$). B7-H4 knocked down inhibited p-JAK2 expression to some extent in Eca109 cells cultured with tocilizumab. These data indicated that B7-H4 silence inhibited JAK2/STAT3 activation independently of IL-6. Taken together, these results showed that B7-H4 repressed IL-6 secretion through JAK/STAT3 inactivation.

Tocilizumab inhibited ESCC growth and tumorigenesis. Next, we investigated the effect of IL-6 on proliferation of ESCC cells. Results in Figure 6 show that, compared with control shRNA, tocilizumab suppressed the proliferation rate of

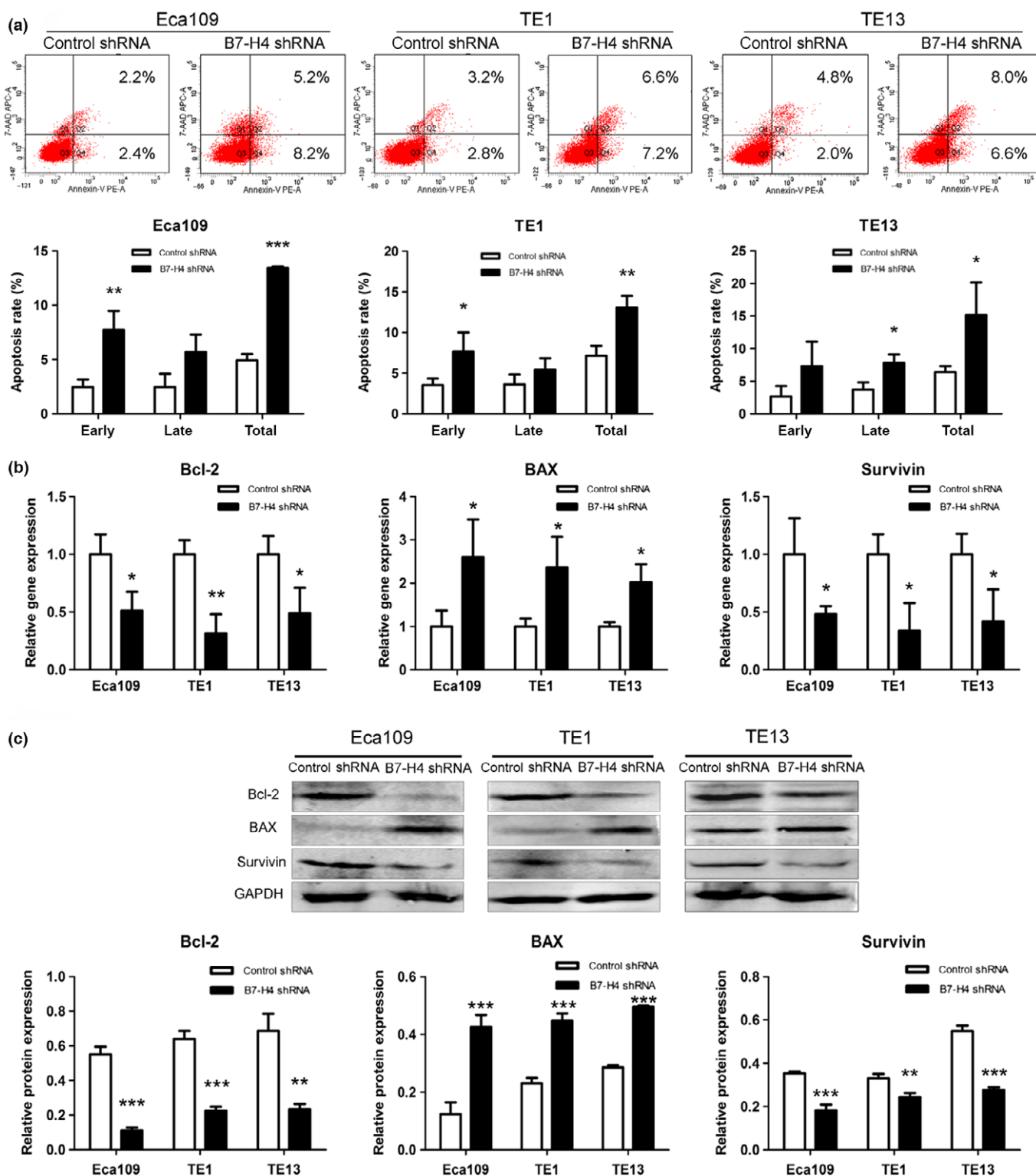


Fig. 2 B7-H4 silencing promoted cell apoptosis in esophageal squamous cell carcinoma cells. (a) Low B7-H4 expression increased the total apoptosis rate of Eca109, TE1, and TE13 cells detected by annexin V-phycoerythrin/7-amino-actinomycin D flow cytometry test kit. Early apoptosis and late apoptosis of B7-H4 silenced cells were also increased to a certain extent. (b, c) Compared with control cells, B7-H4 depletion downregulated apoptosis-inhibiting molecules Bcl-2 and Survivin but upregulated apoptosis-promoting molecule BAX, evaluated by quantitative RT-PCR and Western blot assays. All data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA. PE-A, phycoerythrin/7-amino-actinomycin D.

Eca109, TE1, and TE13 cells pretreated with control shRNA, detected by MTS assay, and decreased the colony numbers of these cells, by colony formation detection. However, compared

with cells pretreated with B7-H4 shRNA, cell proliferation rates and colony numbers of Eca109, TE1, and TE13 cells pretreated with B7-H4 shRNA and tocilizumab were not

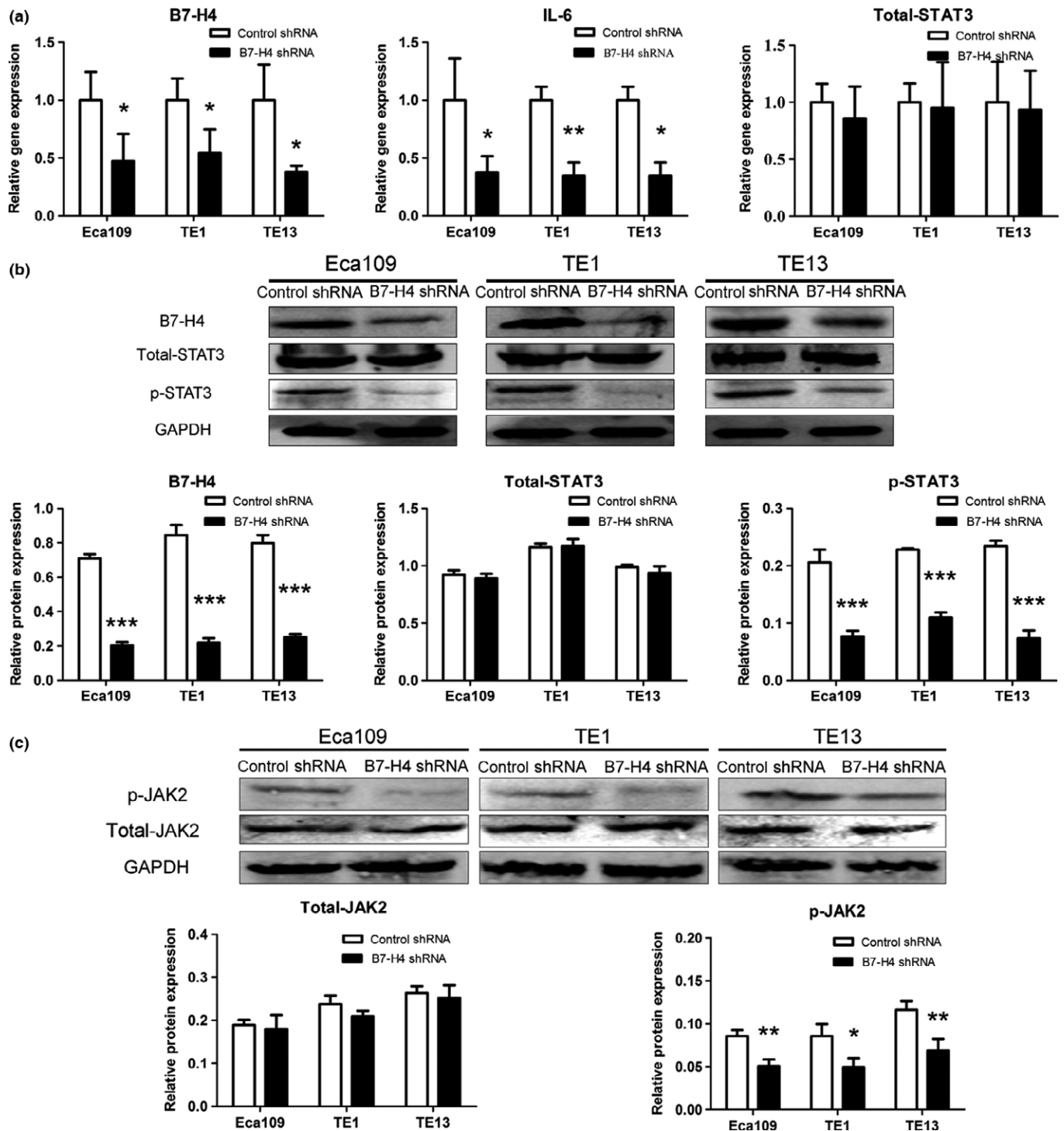


Fig. 3. B7-H4 silence inhibited JAK2/signal transducer and activator of transcription 3 (STAT3) signal pathway activation. (a, b) Quantitative RT-PCR and Western blot assays showed that B7-H4 depletion repressed *IL-6* gene and p-STAT3 protein expression. However, it did not obviously alter total-STAT3 expression at either gene or protein levels. (c) B7-H4 depletion dampened p-JAK2 expression markedly in Eca109, TE1, and TE13 cells ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively) but did not affect total-JAK2 expression, shown by Western blot analysis. All data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA.

obviously affected. Therefore, these data suggest that tocilizumab inhibited the growth of ESCC cells pretreated with control shRNA but not B7-H4 shRNA. That is, IL-6 downregulation induced by B7-H4 silence suppressed ESCC cell growth.

The results of the *in vivo* experiment showed that the tumor volume and tumor weight were all significantly inhibited by 20 mg/kg tocilizumab (Fig. 7a,b).

Collectively, these data indicate that B7-H4 silence suppressed IL-6 secretion through JAK2/STAT3 inactivation. It

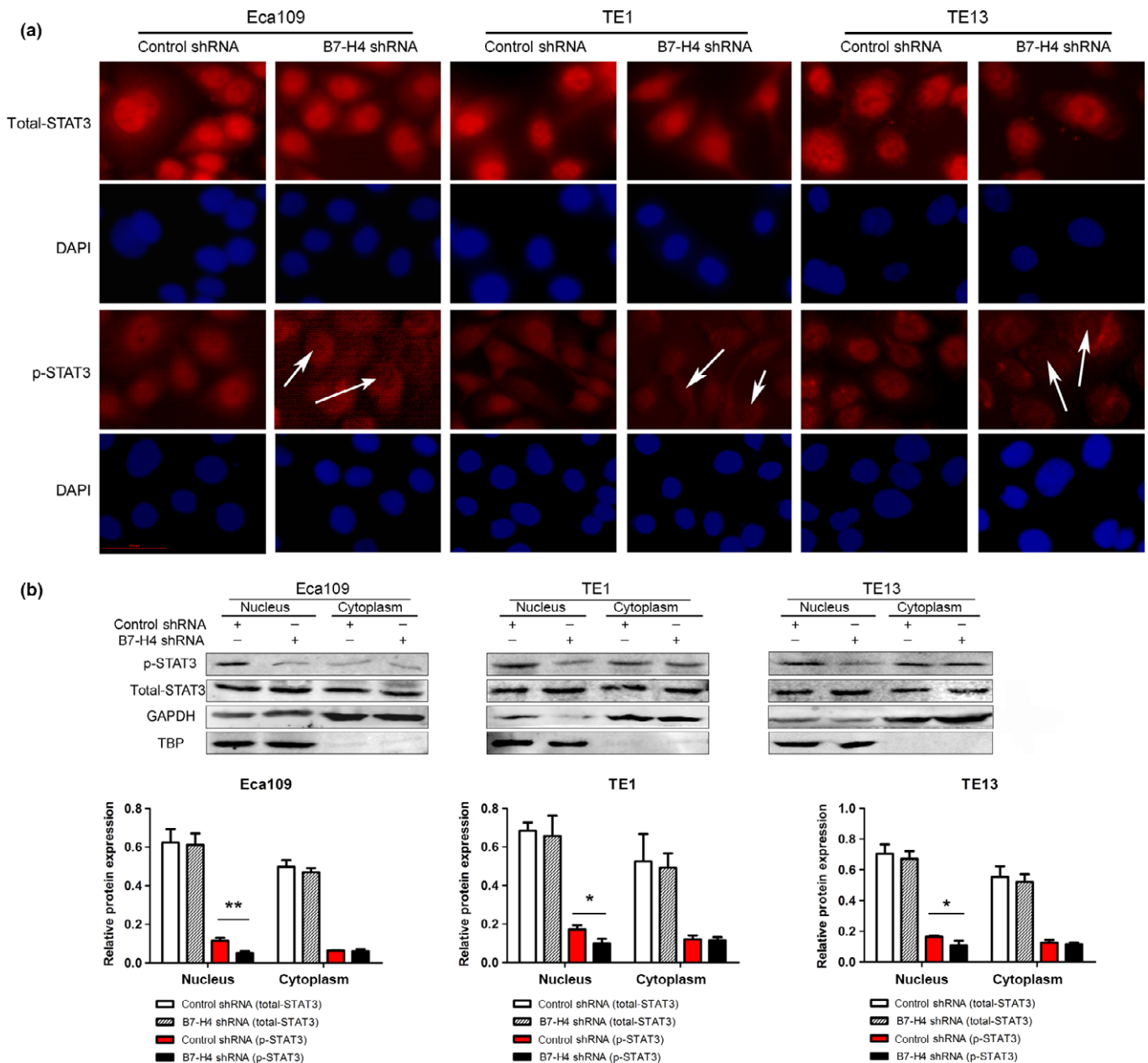


Fig. 4. B7-H4 silence inhibited phosphorylated signal transducer and activator of transcription 3 (p-STAT3) translocation from cytoplasm to nucleus in esophageal squamous cell carcinoma cells. (a) Low B7-H4 expression reduced p-STAT3 location in cell nucleus but did not significantly affect STAT3 location in Eca109, TE1, or TE13 cells evaluated by immunofluorescence staining. The white arrows showed that the fluorescence intensity of the nucleus in B7-H4 shRNA treated cell was decreased compared with that of control shRNA treated cell. (b) Results of Western blot analysis showed that B7-H4 depletion inhibited p-STAT3 location in the nucleus of Eca109, TE1, and TE13 cells with statistical difference ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively). Compared with cells pretreated with control shRNA, p-STAT3 expression in the cytoplasm of cells pretreated with B7-H4 shRNA was not obviously affected. Total STAT3, however, did not change significantly either in cytoplasm or nucleus. The amounts of target proteins in cytoplasm were normalized by GAPDH; protein expression in nucleus was evaluated and normalized by TATA-binding protein (TBP). Data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$ compared with control shRNA.

also inhibited p-STAT3 translocation from cytoplasm to nucleus in ESCC cells. Interleukin-6 reduction induced by B7-H4 silence was necessary for ESCC cell proliferation inhibition. Therefore, we can draw the conclusion that IL-6/STAT3 pathway activation is an important mechanism of B7-H4 promoting ESCC cell proliferation.

Tocilizumab downregulated B7-H4 expression. We then wondered whether B7-H4 expression was affected by IL-6. As Figure 7(c) shows, B7-H4 expression was markedly dampened in tumor tissues of mice treated with tocilizumab compared with

that of control tumor tissues. Results in Figure 7(d) show that tocilizumab also significantly suppressed B7-H4 expression in Eca109, TE1, and TE13 cells ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively). These data suggested B7-H4 expression can be induced by IL-6.

In conclusion, we found that B7-H4 was highly expressed in the three ESCC cell lines, Eca109, TE1, and TE13. B7-H4 silence dampened IL-6 secretion through JAK2/STAT3 pathway inactivation which accounted for ESCC cell proliferation inhibition as well as apoptosis induction. At the same time,

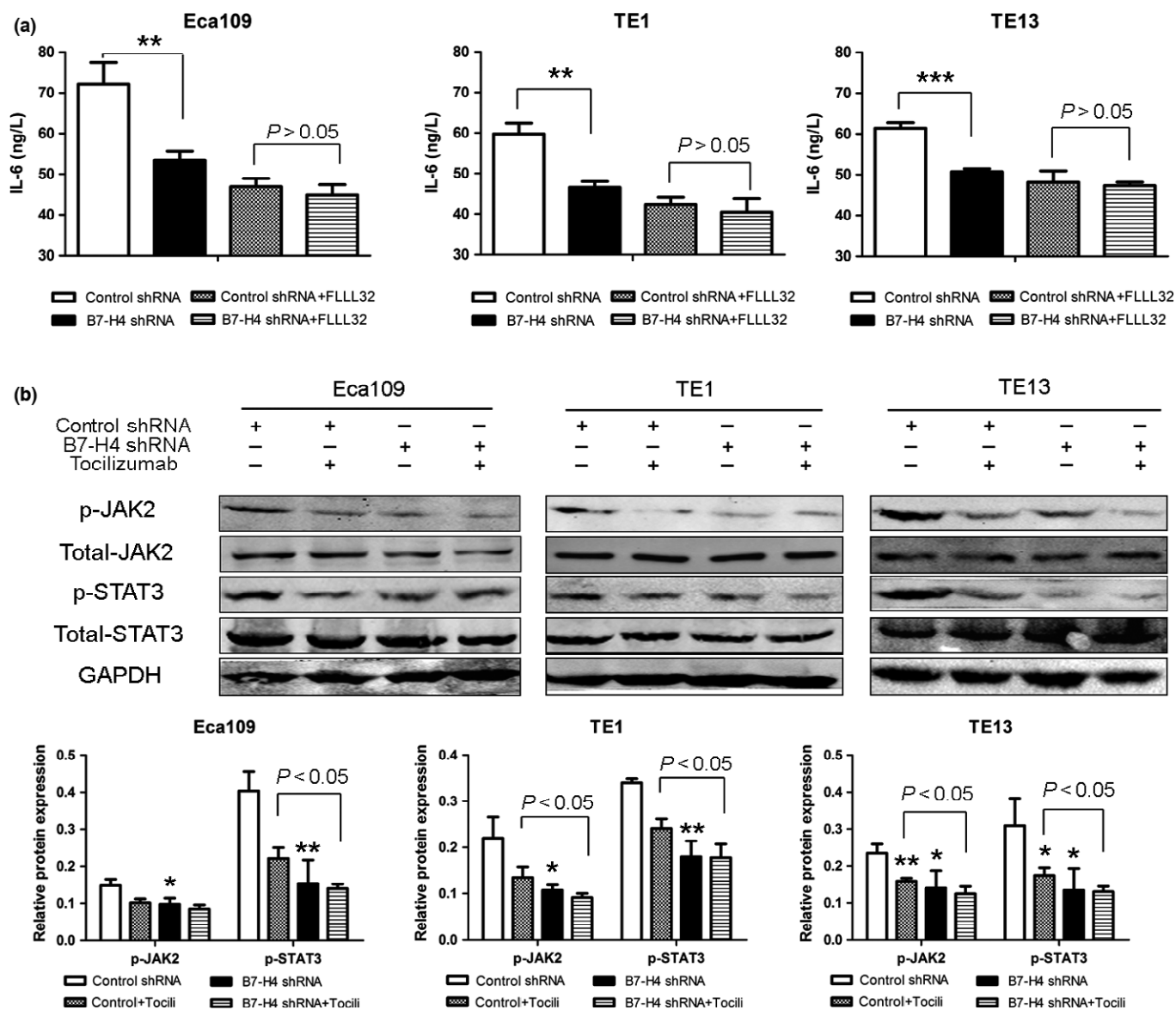


Fig. 5. Interleukin-6 (IL-6) secretion was inhibited by B7-H4 silence through JAK2/signal transducer and activator of transcription 3 (STAT3) inactivation. (a) ELISA results showed that, compared with cells pretreated with control shRNA, B7-H4 silence led to reduction of IL-6 secretion. However, compared with cells pretreated with control shRNA and FLLL32, IL-6 secretion in Eca109, TE1, and TE13 esophageal squamous cell carcinoma cells pretreated with B7-H4 shRNA and FLLL32 was not significantly affected. (b) Western blot analysis indicated that, compared with control cells, B7-H4 silence inhibited p-JAK2 and p-STAT3 expression in Eca109, TE1, and TE13 cells. Compared with cells pretreated with control shRNA and tocilizumab (Tocili), p-STAT3 expression in Eca109, TE1, and TE13 cells and p-JAK2 expression in TE1 and TE13 cells pretreated with tocilizumab and B7-H4 shRNA were also significantly suppressed. Data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA.

tocilizumab was an inhibitor of B7-H4 expression. All these data suggested that B7-H4 promoted ESCC cell growth and tumorigenesis through IL-6/STAT3 positive loopback signal pathway activation. It may be one of the molecular mechanisms of B7-H4 in ESCC development and poor prognosis.

Discussion

In this paper, we chose three ESCC cell lines and found that B7-H4 was highly expressed in all of them. Furthermore, data strongly indicated that B7-H4 silence inhibited cell proliferation, colony formation, and IL-6 secretion, and promoted cell apoptosis. These findings were similar to a previous report on the promoting effect of B7-H4 on prostate cancer cell

proliferation and apoptosis inhibition; the findings also prompted us to investigate whether B7-H4 expression is related to IL-6.

Interleukin-6, one of the most important cytokines widely existing in tumor microenvironment, is an activator of the STAT3 pathway. A member of the STAT family, STAT3 is present in large quantities in cancer cell lines, including head and neck squamous cell carcinoma, breast cancer, ovarian cancer, lung cancer, and melanoma.⁽³²⁾ Accumulating evidence indicates that STAT3 plays an important role during tumor cell proliferation⁽²⁷⁾ and apoptosis inhibition.⁽³³⁾ However, the correlation between B7-H4 and the IL-6/STAT3 signal pathway is unknown. In our study, we found that B7-H4 promoted IL-6 through JAK2/STAT3 activation. Interleukin-6 upregulation

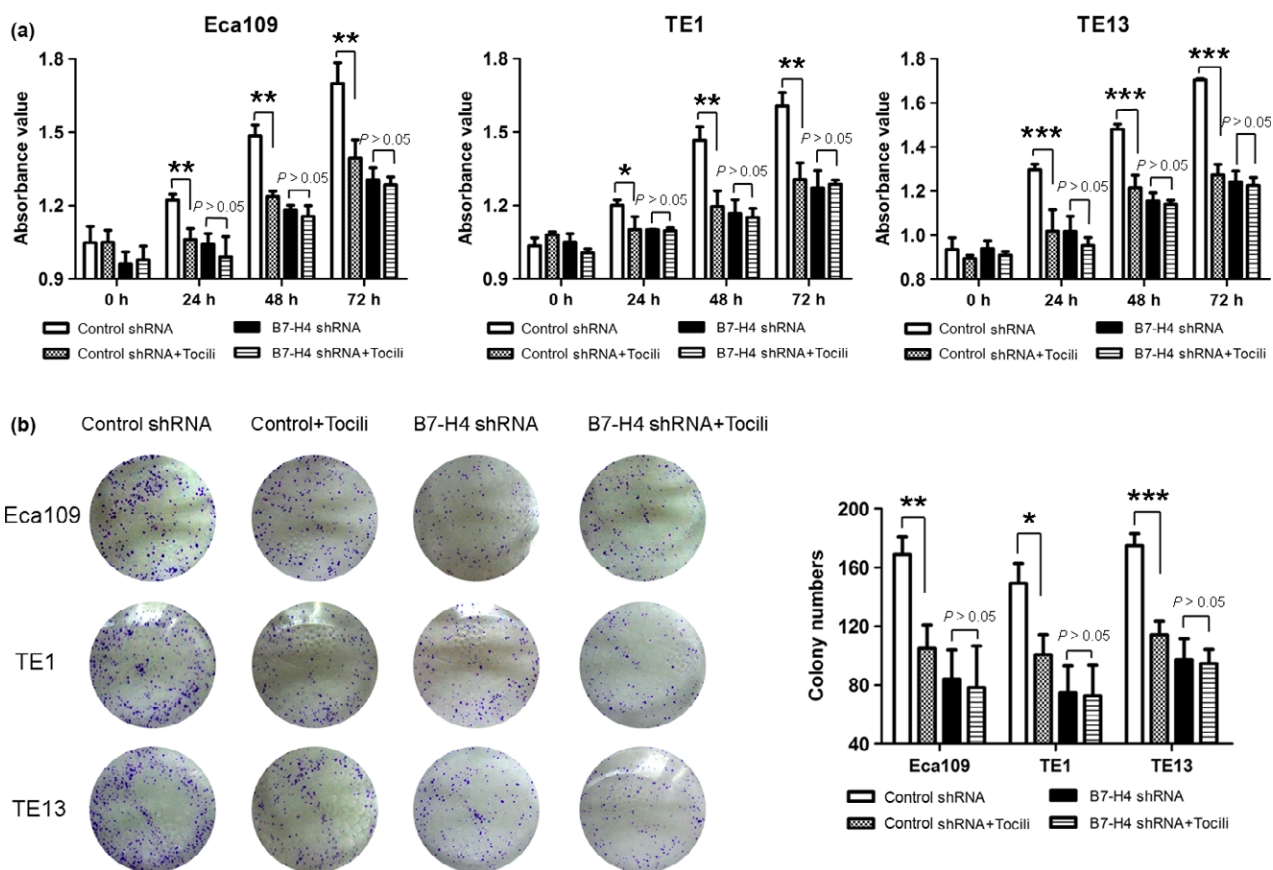


Fig. 6. Interleukin-6 (IL-6) reduction induced by B7-H4 silence inhibited esophageal squamous cell carcinoma cell proliferation. (a) MTS results showed that 200 ng/mL tocilizumab (Tocili) inhibited control Eca109, TE1, and TE13 cell proliferation. However, proliferation of B7-H4 silenced Eca109, TE1, and TE13 cells were not obviously affected by tocilizumab. (b) Tocilizumab (200 ng/mL) inhibited colony formation of control Eca109, TE1, and TE13 cells. However, compared with cells pretreated with B7-H4 shRNA, colony numbers of Eca109, TE1, and TE13 cells pretreated with tocilizumab and B7-H4 shRNA were not markedly changed (all $P > 0.05$). That is, tocilizumab inhibited proliferation and colony formation of control cells but not B7-H4 silenced cells. Data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA.

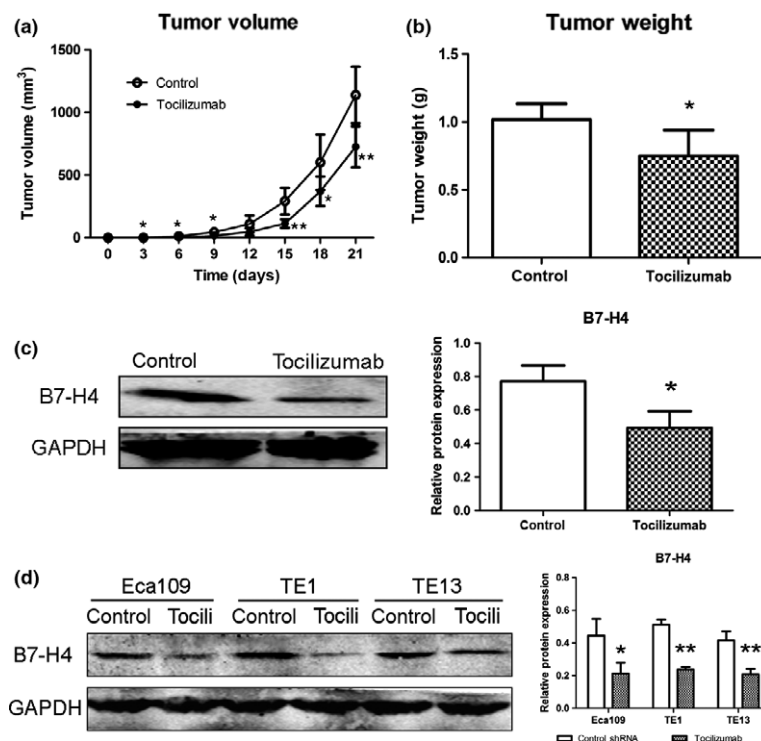


Fig. 7. Tocilizumab inhibited Eca109 cells tumorigenesis and B7-H4 expression. (a, b) Compared with control mice, the tumor volume and tumor weight of mice treated with tocilizumab (20 mg/kg) was significantly inhibited. (c) Compared with control tumor tissues, B7-H4 expression was remarkably suppressed in tumor tissues of mice treated with tocilizumab. $P < 0.05$. (d) Western blot analysis showed that 200 ng/mL tocilizumab (Tocili) inhibited B7-H4 protein expression in Eca109, TE1, and TE13 cells ($P < 0.05$, $P < 0.001$, $P < 0.01$, respectively). Data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control shRNA.

played an important part in B7-H4 facilitating ESCC cell growth and tumorigenesis. However, we were pleased to find that tocilizumab, an IL-6 receptor inhibitor, downregulated B7-H4 expression *in vitro* and *in vivo*. The findings supplemented the previous report that IL-6 induced B7-H4 expression in macrophages.⁽²⁹⁾

Nevertheless, the in-depth mechanism of B7-H4 activating JAK2 still needs to be researched. We speculated that it may be related to the B7-H4 structure. It is well known that oligomerization and cross-phosphorylation are necessary for JAK2 activation.⁽³²⁾ That is, oligomerization of binding sites of B7-H4 is critical to activate JAK2. B7-H4 was diffusely expressed in the cytoplasm and membrane in tumor cells.⁽²⁹⁾ It has the structure of Ig with two identical heavy chains and light chains.⁽³⁴⁾ Although we have not yet determined the binding sites of B7-H4 to target proteins to activate JAK2, we hypothesize that there are two adjacent binding sites on the B7-H4 molecule because of its symmetrical structure. Therefore, we speculate that the Ig structure of B7-H4 is helpful and important to JAK2 activation.

It must be pointed out that we failed to upregulate B7-H4 by overexpressing B7-H4 plasmid in the three cell lines. We also found that B7-H4 expression could not be upregulated by IL-6 (10 ng/mL) in Eca109, TE1, or TE13 cells (data not shown). We considered the possible reasons were as follows. B7-H4 was highly expressed in the three ESCC cells. There might be some kind of negative feedback mechanism in the cells to limit the expression of B7-H4 within a certain range, although the mechanism is not clear. Our results also proved that B7-H4 activated the STAT3 pathway and facilitated IL-6 secretion. In addition, IL-6 was an inducer of B7-H4 expression through combining with IL-6 receptor. That is, the

positive loopback of B7-H4/STAT3/IL-6 kept the expression of B7-H4 at a high level in the ESCC cells. However, STAT3 overactivation could initiate a series of gene expressions. Suppressor of cytokine signaling (SOCS) family members were STAT3 target genes that bind to receptors and further block STAT3 activation by turning off the initial signal. Therefore, we speculated that overactivation of STAT3 of control ESCC cells induced SOCS molecule expression and inhibited further activation of the positive loopback pathway, which led to the expression of B7-H4 not being effectively raised in these ESCC cells.

In all, our present finds indicate that B7-H4 was highly expressed in ESCC cells and played a critical role in facilitating cell proliferation through B7-H4/STAT3/IL-6 positive feedback pathway activation. It provides a new clue to study the mechanism of B7-H4 in ESCC. In-depth studies are still required to understand the underlying mechanism of B7-H4 and to further evaluate whether B7-H4 could be a useful target in ESCC therapy.

Acknowledgments

This work was financially supported by grants from the National Natural Science Foundation of China (Grant No. 81173611), Major Research Project of Hebei Province (Grant No. zd2013045), and the Innovative Funding Project for Graduate Students in Universities from Degree Office of Hebei Education Department.

Disclosure Statement

The authors have no conflict of interest.

References

- Herszényi L, Tulassay Z. Epidemiology of gastrointestinal and liver tumors. *Eur Rev Med Pharmacol Sci* 2010; **14**: 249–58.
- Henry MA, Lerco MM, Ribeiro PW *et al.* Epidemiological features of esophageal cancer: squamous cell carcinoma versus adenocarcinoma. *Acta Cir Bras* 2014; **29**: 389–93.
- Pohl H, Welch HG. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *Natl Cancer Inst* 2005; **97**: 142–6.
- Holmes RS, Vaughan TL. Epidemiology and pathogenesis of esophageal cancer. *Semin Radiat Oncol* 2007; **17**: 2–9.
- Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med* 2003; **349**: 2241–52.
- Siewert JR, Ott K. Are squamous and adenocarcinomas of the esophagus the same disease? *Semin Radiat Oncol* 2007; **17**: 38–44.
- Ajani JA, Barthel JS, Bentrem DJ *et al.* Esophageal and esophagogastric junction cancers. *J Natl Compr Canc Netw* 2011; **9**: 830–87.
- Dong H, Zhu G, Tamada K *et al.* B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999; **5**: 1365–9.
- Wang S, Zhu G, Chapoval AI *et al.* Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* 2000; **96**: 2808–13.
- Chapoval AI, Ni J, Lau JS *et al.* B7-H3: a costimulatory molecule for T cell activation and IFN- γ production. *Nat Immunol* 2001; **2**: 269–74.
- Sica GL, Choi IH, Zhu G *et al.* B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 2003; **18**: 849–61.
- Tseng SY, Otsuji M, Gorski K *et al.* B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med* 2001; **193**: 839–46.
- Brandt CS, Baratin M, Yi EC *et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med* 2009; **206**: 1495–503.
- Prasad DV, Richards S, Mai XM *et al.* B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* 2003; **18**: 863–73.
- Zang X, Loke P, Kim J *et al.* B7x: a widely expressed B7 family member that inhibits T cell activation. *Proc Natl Acad Sci USA* 2003; **100**: 10388–92.
- Sun Y, Wang Y, Zhao J *et al.* B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer* 2006; **53**: 143–51.
- Fauci JM, Straughn JM Jr, Ferrone S *et al.* A review of B7-H3 and B7-H4 immune molecules and their role in ovarian cancer. *Gynecol Oncol* 2012; **127**: 420–5.
- Zang X, Thompson RH, Al-Ahmadie HA *et al.* B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. *Proc Natl Acad Sci USA* 2007; **104**: 19458–63.
- Quandt D, Fiedler E, Boettcher D *et al.* B7-h4 expression in human melanoma: its association with patients' survival and antitumor immune response. *Clin Cancer Res* 2011; **7**: 3100–11.
- Arigami T, Uenosono Y, Ishigami S *et al.* Clinical significance of the B7-H4 coregulatory molecule as a novel prognostic marker in gastric cancer. *World J Surg* 2011; **35**: 2051–7.
- Mugler KC, Singh M, Tringler B *et al.* Expression in a range of breast pathology: correlation with tumor T-cell infiltration. *Appl Immunohistochem Mol Morphol* 2007; **15**: 363–70.
- Jung SG, Choi KU, Lee SD *et al.* The relationship between B7-H4 expression and clinic pathological characteristics in clinical stage T1 conventional renal cell carcinoma. *Korean J Urol* 2011; **52**: 90–5.
- Chen LJ, Sun J, Wu HY *et al.* B7-H4 expression associates with cancer progression and predicts patient's survival in human esophageal squamous cell carcinoma. *Cancer Immunol Immunother* 2011; **60**: 1047–55.
- He C, Qiao H, Jiang H *et al.* The inhibitory role of B7-H4 in antitumor immunity: association with cancer progression and survival. *Clin Dev Immunol* 2011; **2011**: 695834.
- Cheng L, Jiang J, Gao R *et al.* B7-H4 expression promotes tumorigenesis in ovarian cancer. *Int J Gynecol Cancer* 2009; **19**: 1481–6.
- Qian Y, Hong B, Shen L *et al.* B7-H4 enhances oncogenicity and inhibits apoptosis in pancreatic cancer cells. *Cell Tissue Res* 2013; **353**: 139–51.
- Demaria M, Camporeale A, Poli V. STAT3 and metabolism: how many ways to use a single molecule? *Int J Cancer* 2014; **135**: 1997–2003.

- 28 Dvorak K, Chavarria M, Payne CM *et al.* Activation of the interleukin-6/STAT3 antiapoptotic pathway in esophageal cells by bileacids and low pH: relevance to Barrett's Esophagus. *Clin Cancer Res* 2007; **13**: 5305–13.
- 29 Kryczek I, Zou L, Rodriguez P *et al.* B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J Exp Med* 2006; **203**: 871–81.
- 30 Kryczek I, Wei S, Zhu G *et al.* Relationship between B7-H4, regulatory T cells, and patient outcome in human ovarian carcinoma. *Cancer Res* 2007; **67**: 8900–5.
- 31 Peng HX, Wu WQ, Yang DM *et al.* Role of B7-H4 siRNA in proliferation, migration, and invasion of LOVO colorectal carcinoma cell line. *Biomed Res Int* 2015; **2015**: 326981.
- 32 Abroun S, Saki N, Ahmadvand M *et al.* STATs: an old story. Yet mesmerizing. *Cell J* 2015; **17**: 395–411.
- 33 Alas S, Bonavida B. Rituximab inactivates signal transducer and activation of transcription 3 (STAT3) activity in B-non-Hodgkin's lymphoma through inhibition of the interleukin 10 autocrine/paracrine loop and results in down-regulation of Bcl-2 and sensitization to cytotoxic drugs. *Cancer Res* 2001; **61**: 5137–44.
- 34 Jeon H, Vigdorovich V, Garrett-thomson SC *et al.* Structure and cancer immunotherapy of the B7 family membrane B7x. *Cell Rep* 2014; **9**: 1089–98.