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

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Eosophageal 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-H1,7–9 B7-H2,10 B7-H3,11 B7-H4,12 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, breast,20 kidney,21 and esophagus.22 It has been proved that B7-H4 plays an important role in regulating adaptive immune response by inhibiting the proliferation, activation, and cytolytic function of T cells, and host innate immune response by inhibiting the growth of neutrophil progenitors.23 In addition, Cheng et al.24 found that B7-H4-deficient mice showed lower ovarian tumorigenicity and Qian et al.25 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), a cytokine 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% CO\(_2\)/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’-GTTGACATATAATGCGACGCTAGACCTT-3’. For the transfection process, approximately 5 \(\times\) 10\(^5\) cells were seeded in 6-well plates. After 24 h of culture, 2 \(\mu\)g transfect DNA and 4 \(\mu\)L 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 \(\mu\)L [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)]-2H-tetrazolium, inner salt (MTS; Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 without FBS was added into each well to incubate for 200 min. After the medium was exchanged by RPMI-1640 with 10% FBS, followed by 48 h of incubation. The cells were then harvested for analysis.

**Cell apoptosis.** 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–phycocerythrin/7-amino-actinomycin D apoptosis detection kit (BD, San Jose, CA, USA). In brief, 1 \(\times\) 10\(^6\) cells for every sample were harvested, then washed with PBS and incubated with 5 \(\mu\)L annexin V–phycocerythrin, and 5 \(\mu\)L 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 \(\mu\)g) 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 \(\mu\)L reaction mixture containing cDNA product (2 \(\mu\)g), specific primers (0.2 \(\mu\)mol/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’-TAATGCAGCCGCTTGTGACG-3’ (sense) and 5’-TCAGAGTTCTCCTCCCCCA-3’ (antisense); IL-6, 5’-CCATCCAGTTGCTCTTTTGAG-3’ (sense) and 5’-CTCTCTTCCGGACTTGTGAA-3’ (antisense); and GAPDH, 5’-CCTGTGAACGCTGCTTGG-3’ (sense) and 5’-TGCTTGTAACACCTTGG-3’ (antisense). The PCR was run previously.\(^{31}\) The transfer times were: 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, and p-STAT3; and 2 h for JAK2 and p-JAK2. The amounts of target proteins were detected by a Bicinchoninic Acid kit (Sigma-Aldrich).

**Immune fluorescence staining.** Cells harvested were fixed in 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 \(\mu\)M FLLL32 (Selleck Chemicals, Houston, Texas, USA) and 5 \(\mu\)M IL-6. For the IL-6 detection by ELISA, the cells were treated with IL-6 and FLLL32 for 48 h. The supernatant was collected and measured by the CTG-58G kit (One Step ELISA Kit; Wuxi Jilong Bio Technology, Shanghai, China). The absorbance at 490 nm was measured.
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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, $5 \times 10^6$ 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 collected for IL-6 detection following ELISA kit instructions. 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 collected for IL-6 detection following ELISA kit instructions.

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.

**Statistical analysis.** Results were reported as mean ± 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 need 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 on 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. 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-related protein expression, such as Bcl-2 members. 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. Accordingly, we further investigated the location of STAT3 by immunofluorescence 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.
significantly in cells pretreated with B7-H4 shRNA compared with those 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
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 reduced.

Fig. 2. B7-H4 silence 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 ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control shRNA. PE-A, phycoerythrin/7-amino-actinomycin D.
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
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,
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. Accumulating evidence indicates that STAT3 plays an important role during tumor cell proliferation and apoptosis inhibition. 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 ± 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 ± 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. (20) 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. Suppression 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 findings 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.

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Disclosure Statement

The authors have no conflict of interest.
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