

## Original Article

# The clinical significance and contribution of the co-stimulatory molecule B7-H1 in human esophageal cancer

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**Abstract:** Object: The study aims to investigate the clinical significance and contribution of the co-stimulatory molecule B7-H1 in human esophageal cancer tissues. Methods: Immunohistochemistry and western blot were used to analyze the expression levels of B7-H1 in human esophageal cancer tissues and cells. The associations between the expression of B7-H1 and other variables, including clinical parameters and prognosis, were explored using the fourfold and contingency table chi square tests respectively. After the expression of B7-H1 in human esophageal cancer cells was disrupted by an siRNA, the proliferation and invasive activity of Eca-109 were tested using the CKK8 and Transwell assays respectively. Results: B7-H1 was highly expressed in esophageal cancer cells and tissues, and its expression level had a significant and positive correlation with the depth of tumor invasion ( $P < 0.05$ ). The survival rate was much lower in patients with higher B7-H1 expression levels ( $P < 0.05$ ). A B7-H1-knockdown model in Eca-109 cells was constructed by lentiviral transfection and RNAi, and downregulation of B7-H1 remarkably inhibited the proliferation and invasion of esophageal cancer cells. Conclusion: Negative co-stimulatory molecule B7-H1 is regarded as an important bio-marker for the diagnosis and prognosis of esophageal cancer.

**Keywords:** B7-H1, esophageal cancer, proliferation, invasion

## Introduction

Tumor immune escape is a molecular mechanism that happens during the occurrence, invasion, and metastasis process of tumors. Tumor cells do not possess the appropriate “danger signals” to alert the immune system to the presence of a foreign cell [1], so the immune system will ignore or tolerate a developing tumor because the tumor cells are too similar to the normal cells from which they are derived [2]. Therefore, tumor cells are able to counteract the attack of immune effector cells and escape from the surveillance of immune systems. Co-stimulatory molecules, which consist of super families, namely B7/CD28 and TNF/TNFR [3], play an important role in the suppression of immune responses to tumor cells. Recently, a study reported that co-stimulatory molecules within B7 families, such as B7-H1, B7-H3, and B7-H4, are present in many human tumor tissues and responsible for the downreg-

ulation of anti-tumor immune responses modulated by T cells [4, 5]. B7-H1 is expressed on antigen-presenting cells (APCs), activated T cells and B cells, macrophages, placental trophoblasts, and cortical epithelial cells of the cardiac endothelium and thymus, and has been detected in many human tumor tissues [6]. The expression level of B7-H1 is significantly up-regulated compared with normal tissues and is highly related to clinicopathological features and postoperative prognosis [7-9].

In the past few years, breakthroughs have been made in tumor immunotherapy by using function-blocking antibody “checkpoint” molecules such as CTLA-4 and PD-1/B7-H1. Specifically, targeted B7-H1 has achieved good curative results in the immunotherapy of melanoma, lung cancer, and other tumors [10, 11], providing a new immunotherapy with important scientific value and offering a promising prospect for clinical application. *Science's* editors chose

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cancer immunotherapy as the top breakthrough of the Year for 2013 [12]. In this study, we analyzed the expression of the co-stimulatory molecule B7-H1 in human esophageal cancer cells and tissues to explore its clinical significance and potential contribution. By applying RNA interference technology, we further investigated the effect of the downregulated expression of B7-H1 on the proliferation and invasion abilities of the esophageal cancer cell line Eca-109.

### Materials and methods

#### *Specimens, cells, reagents and antibodies*

The specimens were selected from 198 patients undergoing esophageal cancer surgery in our hospital from March 2013 to March 2014. A total of 152 male patients and 46 females were included. The patients' ages ranged from 37 to 78 years, with average an age of 59. Meanwhile, 20 normal esophageal tissues were collected as controls. None of the patients received any radiotherapy or chemotherapy before surgery, and we collected detailed clinical and follow-up data. Postoperative tissue specimens were fixed with 10% formaldehyde and embedded in paraffin for further analyses.

The human esophageal cancer cell line Eca-109 and human normal esophageal epithelial cells BNCC337729 were purchased from Shanghai Enzyme Biotech Co., Ltd. We also obtained the following: Rabbit antihuman B7-H1 polyclonal antibody (NBP1-03220, Novus Biologicals, 1:200); Secondary antibodies used in immunohistochemistry: Horseradish peroxidase (HRP) marker rat/rabbit universal secondary antibody (Ready to use, DAKO, Glostrup, Denmark); Citrate Antigen Retrieval solution (MVS-0066, Fuzhou Maxim); BSA (Gene-tech, Co. Ltd.), Cyanobacterium cell fast stain solution (XTS-1090, Fuzhou Maxim); DAB color-developing agent (Dako, Glostrup, Denmark); Permout TM Mounting Medium (DAB-0033, Fuzhou Maxim); Antibody for flow cytometry: PE rat anti-human B7-H1 antibody (BD Biosciences, San Jose, CA, USA); PE-mouse Ig G1 (R&D Systems Inc., Minneapolis, USA).

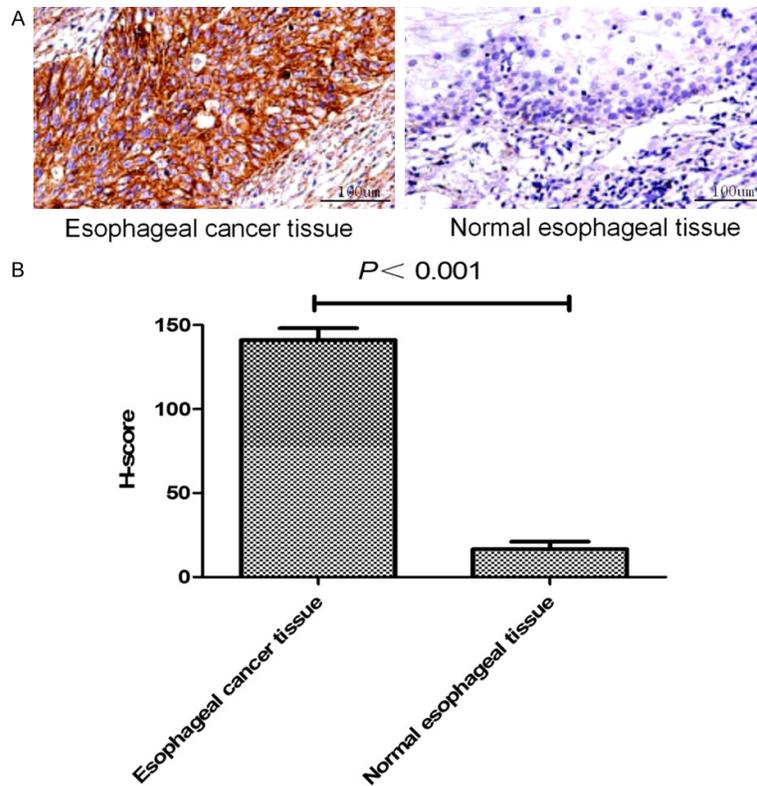
The siRNA sequence for B7-H1 (5'-GAGCAGG-GCTTGTGGATGTG-3') and the nonsense sequence (5'-TTCTCCGAACGTGTACAGT-3') were synthesized by Shanghai Jikai Gene Technology Co., LTD.

#### *Method*

*Immunohistochemistry (IHC):* The paraffin sections were dewaxed and gradient alcohol hydrolyzed. The detailed process was as follow: xylene I, 10 min; xylene II, 10 min; xylene III, 10 min; 100% anhydrous ethanol I, 3 min; 100% anhydrous ethanol II, 3 min; 95% ethanol I, 3 min; 95% Ethanol II, 3 min; 75% Ethanol, 3 min. The treated slices were rinsed under tap water for 10 min, rinsed again with distilled water for 5 min, heat treated in a water bath for 30 min, then we used a citrate buffer for antigen retrieval, then we immersed the slices in distilled water for 5 min, and then we added 3% H<sub>2</sub>O<sub>2</sub> solution blocked endogenous peroxides. The enzyme was incubated at room temperature for 20 min and washed with distilled water. The 5% BSA blocked non-specific binding sites were incubated at room temperature for 15 min. Then we rinsed the specimens for 5 min with PBS each time and repeated this three times. Then we added the primary antibodies and placed them at 4°C overnight. Then we took out the slices, washed them with PBS for 5 min and repeat this three times. Then we added the Rabbit Anti-Mouse IgG (H + L)-HRP, incubated it at 37°C for 30 min, washed with PBS for 5 min and repeated this 3 times, and then we removed the excess secondary antibodies, stained them with a DAB reagent, double stained them with hematoxylin and differentiated them with 1% hydrochloric acid-alcohol. After dehydration in gradient ethanol (75% ethanol 3 min, 95% ethanol I 3 min, 95% ethanol II 3 min, 100% anhydrous ethanol I 3 min, 100% anhydrous ethanol II 3 min), the slice specimens were dried and sealed with a neutral resin. The immunohistochemical staining assessment was evaluated by the H-score method [13]: H-score = (% tumor cells unstained × 0) + (% tumor cells stained weak × 1) + (% tumor cells stained moderate × 2) + (% tumor cells stained strong × 3). The H-scores ranged from 0 (100% negative tumor cells) to 300 (100% strongly stained tumor cells). The nuclear staining of B7-H1 was considered positive if there was any B7-H1 staining in the nucleus of the esophageal cancer cells.

*Cell culture:* The human esophageal cancer cell line Eca-109 and the human normal esophageal epithelial cells BNCC337729 were cultured in 10% FBS RPMI 1640 medium or DMEM high-glucose medium; all the cells were kept at

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**Figure 1.** Expression of B7-H1 in esophageal carcinoma and normal esophageal tissues ( $\times 200$ ).

37°C and 5% CO<sub>2</sub>. The cells were routinely passaged, and the liquid was changed regularly.

**Cell transfection:** The Eca-109 cells line logarithmic growth phase was trypsinized in order to create the cell suspension; then the cell suspension (approximately  $5 \times 10^4$  cells) was inoculated into 6-well plates to reach the cell fusion degree at about 30%. The recombinant RNA interference lentivirus LV-RNAi-B7-H1 and the negative control lentivirus LV-NC (Lentivirus Negative Control, LV-NC) were transfected respectively, and the Eca-109 cells without any transfection were set as the control group.

**Western blot:** The cells were harvested, and an appropriate amount of RIPA lysis buffer containing protease inhibitors was placed on ice for 30 min. Then, the lysate was transferred to an EP tube, centrifuged at 12,000 r/min at 4°C for 15 minutes, and then we transferred the protein supernatant to a new EP tube. The concentration of protein was measured using a BCA kit. After separation by 8%, 10%, and 15% SDS-PAGE, aliquots of the protein were transferred to PVDF membrane using a semi-dry method, blocked, added to the primary anti-

body and incubated overnight at 4°C on a shaker; and then we washed the membrane with TPBS and added a secondary antibody. The protein expression was detected with an ECL detection system (KPL Inc, USA) and exposed on an X-ray film. The optical densities of the protein bands on the X-ray film were quantitatively analyzed with Quantity one software 4.6.2 (Bio-Rad). GAPDH was used as a loading control.

**CCK cell proliferation:** The stable cell lines in each group were digested with trypsin, and single cell suspensions were prepared with the complete medium. After counting, the cell density was adjusted. Cells were inoculated into a 96-well plate with the inoculation density of 2000 cells/well. The plate was incubated at 37°C and 5% CO<sub>2</sub> for 4 hours. Each experimental

group was performed in quadruplicate. 90 µL fresh complete medium and 10 µL CCK-8 solution was added into each well after 0 h, 12 h, 24 h, 48 h, 72 h, and 96 h of incubation. The absorbance at 450 nm was measured with a microplate reader after 3 hours' incubation. The mean value of A450 in each group was plotted on the ordinate and the time was plotted on the abscissa to observe the growth of the cells in each group.

**Transwell invasion assay in vitro:** The cells in the logarithmic growth phase were collected and digested with trypsin digestion; the medium was discarded by centrifugation after digestion. The cells were washed three times in a 1% FBS RPMI1640 medium and counted and re-suspended in a 1% FBS RPMI1640 medium to reach a final cell density of  $5 \times 10^5$  cells/ml.  $2 \times 10^4$  cells were transferred to the top chamber of the Transwell and a 1% FBS RPMI1640 medium was added also. Then we added 1.5 ml of conditioned medium to the lower chamber of the Transwell plates and incubated it at 37°C, 5% CO<sub>2</sub> for 6 h. Cotton swabs were used to remove the un-migrated cells, and the cham-

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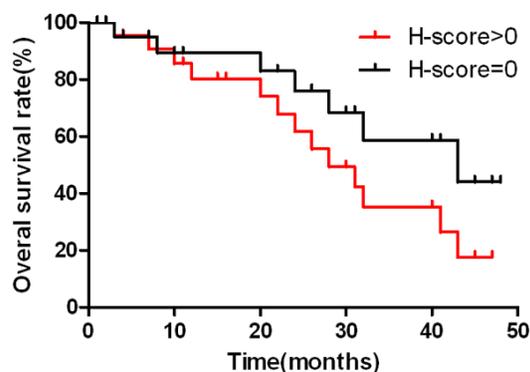
**Table 1.** B7-H1 Relationships between the expression levels of B7-H1 in esophageal cancer and clinicopathological parameters

Clinicopathological Parameters	Cases	B7-H1		$\chi^2$	P
		H-score = 0	H-score > 0		
Gender				0.3590	0.5491
Male	152 (76.8%)	28	124		
Female	46 (23.3%)	6	40		
Age				0.4389	0.5077
< 60	102 (51.5%)	14	56		
≥ 60	96 (48.5%)	20	108		
Tumor Size (cm)				0.3045	0.5811
< 3.5	70 (35.4%)	14	56		
≥ 3.5	128 (64.6%)	20	108		
T Stage (T)				4.9470	0.0261
T1 + T2	70 (35.4%)	20	50		
T3 + T4	128 (64.6%)	14	114		
Lymph Node Metastasis (N)				0.0082	0.9277
No	130 (65.7%)	22	108		
Yes	68 (34.3%)	12	56		
Distant Metastasis (M)				1.3240	0.2498
No	186 (93.9%)	34	152		
Yes	12 (6.1%)	0	12		
TNM Stage				0.9872	0.3204
I	10 (5.0%)	6	4		
II	114 (57.6%)	14	100		
III	62 (31.3%)	14	48		
IV	12 (6.1%)	0	12		

brane was merged in the stain and incubated at room temperature for 2 hours. Then we took out the chamber and washed it with 1 × PBS solution. The chamber was then placed on the microscope stage to enable the counting of the invaded cells.

### Statistics analysis

The statistical analysis of the data was performed using Graph Pad Prism 5.0 statistical software. A paired Student's *t*-test, the Wilcoxon signed rank test, or the survival analysis were used where appropriate. A *P*-value of < 0.05 was considered statistically significant. The differences in the rates of the two groups and the correlation analysis of the B7-H1 expression levels were statistically analyzed using a fourfold or contingency table ( $\chi^2$ ) test.



**Figure 2.** The relationship between the expression of B7-H1 and the prognosis of esophageal cancer.

bers were soaked in methanol for 15 minutes at room temperature. After fixation was completed, the chambers were removed and inverted to thoroughly air dry the porous membrane. The dried chambers were placed in Transwell plates, and 1.5 ml of crystal violet dye solution were added to the lower chamber. The mem-

## Results

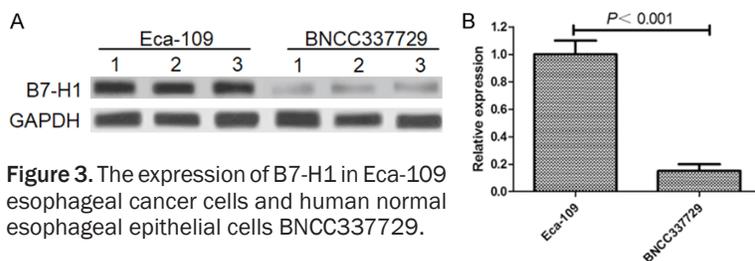
### The expression of B7-H1 in esophageal carcinoma and normal esophageal tissue

The expression of B7-H1 was mainly on the membranes and cytoplasm of the tumor cells, but it was low or absent in normal esophageal tissue. The H-score of B7-H1 in the esophageal cancer tissues was significantly higher than in normal esophageal tissue ( $P < 0.001$ ) (Figure 1).

### The relationship between the expression level of B7-H1 in esophageal cancer tissues and the clinicopathological parameters

The rate of B7-H1 staining in patients with an H-score > 0 in the T3 + T4 group was significantly higher than the rate in the T1/T2 group ( $P < 0.05$ ). There was no significant difference in the patients' gender, age, tumor size, lymph node metastasis, distant metastasis, or TNM

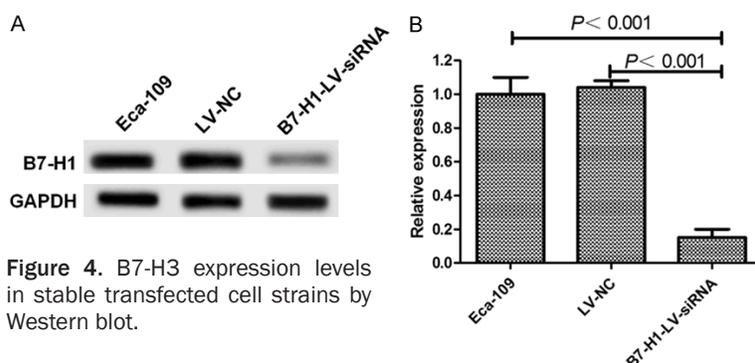
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**Figure 3.** The expression of B7-H1 in Eca-109 esophageal cancer cells and human normal esophageal epithelial cells BNCC337729.

H-score > 0 group ( $\chi^2 = 8.8285$ ,  $P = 0.0030$ ) (**Figure 2**).

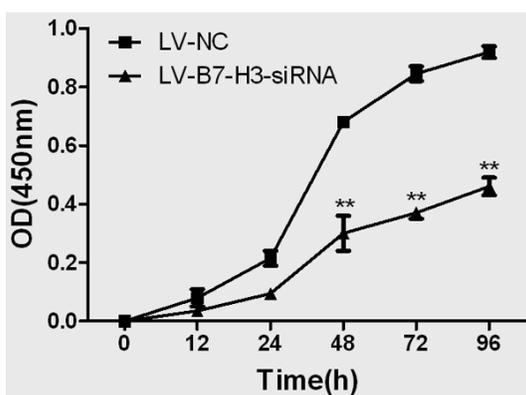
The expressions of B7-H1 in Eca-109 esophageal cancer cells and human normal esophageal epithelial cells BNCC337729



**Figure 4.** B7-H3 expression levels in stable transfected cell strains by Western blot.

The Western blot showed that B7-H1 was highly expressed in esophageal cancer cells Eca-109, and its expression level was significantly higher than it was in normal human esophageal epithelial cells BNCC337729 ( $P < 0.001$ ) (**Figure 3**).

Construction of stable down-regulated B7-H1 esophageal cancer cells



**Figure 5.** The proliferation ability of stable transfected cell strains by CCK-8 analysis (\*\*compared with LV-NC,  $P < 0.05$ ).

Western blot analysis showed that the expression level of B7-H3 protein in Eca-109 cell line that were stably transfected with LV-B7-H3-siRNA was significantly lower than those of the Eca-109 cell line stably transfected with LV-NC and the un-transfected group, suggesting a successful construction of stable down-regulated B7-H1 esophageal cancer cells (**Figure 4**).

Proliferation ability of stable transfected cell strains by CCK-8 analysis

To further compare the effect of targeted intervention with B7-H3 expression on the proliferation of esophageal cancer cell Eca-109, we performed a CCK-8 proliferation analysis at different time points using Eca-109 cells that were stably transfected with LV-B7-H3-siRNA and LV-NC. The results showed that the absorbance values of the Eca-109-LV-B7-H3-siRNA group were significantly lower than those of the Eca-109-LV-NC group at 48 h, 72 h, and 96 h, suggesting that the downregulation of B7-H3 in the Eca-109 cell line can significantly inhibit cell proliferation (**Figure 5**).

The effect of B7-H3 on the invasion and metastasis of esophageal cancer cells by transwell

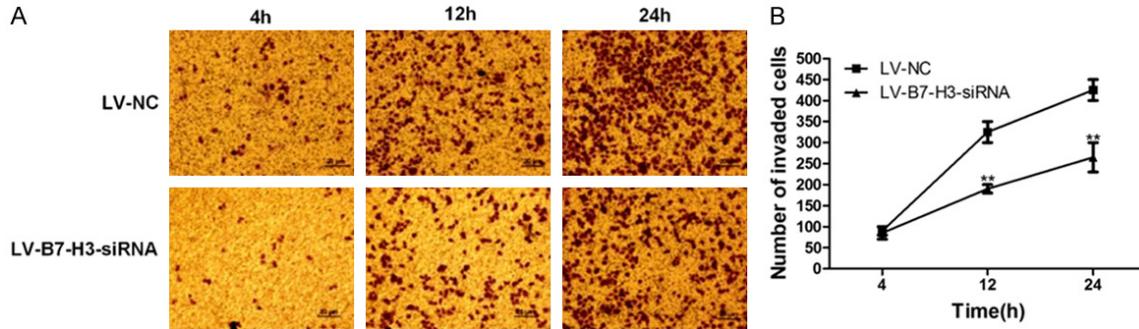
In order to confirm that B7-H3 is able to promote the invasion of esophageal cancer cells,

staging between the two groups ( $P > 0.05$ ) (**Table 1**).

The relationship between the expression of B7-H1 and the prognosis of esophageal cancer

All the patients enrolled in the study were followed up for 4 years. The four-year survival rate was 45.12% (74/164) and 17.65% (6/34) respectively in the B7-H1 staining H-score = 0 group and H-score > 0 group. The 4-year survival rate of the B7-H1 staining H-score = 0 group was significantly higher than that of

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**Figure 6.** The effect of B7-H3 on the invasion, migration and metastasis of esophageal cancer cells by Transwell (\*\*compared with LV-NC,  $P < 0.05$ ).

Transwell experiments were used to compare the cell invasion, migration and metastasis ability of Eca-109 cell lines that were stably transfected with LV-B7-H3-siRNA and LV-NC. The results showed that in the Eca-109 cell line of the LV-B7-H3-siRNA group, and the number of transmembrane cells at 12 h and 24 h was significantly lower than that of the Eca-109 cell line in LV-NC group (Figure 6).

### Discussion

Esophageal cancer is one of the most common malignant tumors of the upper digestive tract. The mortality rate is ranked fifth and eighth in males and females, respectively [14]. In recent years, although various comprehensive treatment methods such as surgery, radiotherapy, chemotherapy, and biological therapy have been widely used in the treatment of esophageal cancer, the prognosis and the 5-year survival rate of patients are still low [15]. Therefore, an in-depth study of the molecular mechanism of esophageal cancer development and seeking new molecular targets for the diagnosis and prognosis of esophageal cancer have important clinical value.

The co-stimulatory molecule B7-H1 is a newly discovered and important member of the B7 family [16]. Previous studies have found that B7-H1 is abnormally expressed in various tumors, such as gastric cancer, colorectal cancer, and renal cancer, and its expression level is closely related to multiple clinical pathological parameters and the prognosis of patients [17-19]. Given that the receptor of B7-H1 molecule is not yet clear, the role and mechanism of B7-H1 molecule in tumor immune response needs to be explored. In this study, we detected

the expression of B7-H1 in esophageal cancer tissue using immunohistochemistry and found that B7-H1 was highly expressed in esophageal cancer tissue. The expression level of B7-H1 was significantly correlated with the depth of tumor invasion, and the postoperative overall survival rate of patients with high expression of B7-H1 was significantly lower than those with a low B7-H1 expression. The findings suggested that B7-H1 acts as a negative regulatory factor in tumor immune response and participates in the tumor immune escape mechanism, and it can be used as a potential indicator for tumor diagnosis and prognosis.

In this study, Western blot was used to detect a high expression of B7-H1 in the human esophageal cancer cell line Eca-109, which is consistent with previous studies indicating that B7-H1 expression can be detected in many tumor cell lines [20]. Liu et al. [21] found that the expression level of B7-H1 in prostate cancer tissues is positively correlated with the expression of the cell proliferation marker Ki-67. Our further analyses showed that RNA interference reduced the expression of B7-H1 in prostate cancer cell line PC-3, and the adhesion, migration, and invasion of tumor cells significantly decreased, suggesting that the abnormal expression of B7-H1 on tumor cells participates in the regulation of T cells, mediates tumor immune responses and is involved in regulating some of the biological properties of tumor cells. Some co-stimulatory molecules can act as both receptors and ligands, and when these membrane surface molecules are stimulated by above mentioned receptors or ligands, they will transmit retrograde signals to cells, resulting in changes to cells' biological characteris-

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tics. We constructed an Eca-109 esophageal cancer cell line stably expressing B7-H1-siRNA through a lentivirus vector and performed a cell proliferation assay and a Transwell invasion and metastasis assay. The results showed that downregulated B7-H1 expression in the esophageal cancer cell line significantly inhibited cell proliferation, invasion and metastasis, suggesting that B7-H1 can also promote the occurrence and development of tumors by regulating the biological behavior of esophageal cancer cells.

In summary, B7-H1 can be used as an important biological indicator for the diagnosis and prognosis of esophageal cancer, and as a potential target for the diagnosis and immunotherapy of esophageal cancer.

### Disclosure of conflict of interest

None.

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