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Original Article

B7-H3 expression associates with tumor invasion and patient's poor survival in human esophageal cancer

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Abstract: B7-H3, a member from B7-family co-stimulatory ligands, plays an important role in adaptive immune responses. In addition, recent studies also demonstrated that B7-H3 could be highly expressed in various types of human cancers, and its expression level was significantly associated with cancer patients' clinicopathological parameters and postoperative prognoses. As of now, the exact role of B7-H3 expression in human esophageal cancer still remains elusive. In the present study, we characterized the B7-H3 expression in the human esophageal cancer cell line Eca-109 and TE-1, and in 174 cases of human esophageal cancer tissues, and to analyze its clinical implications and its correlation to T cell infiltration. By using the RNA interference method to down-regulate the B7-H3 expression in human esophageal cancer cell line Eca-109, we further studied the contribution of high B7-H3 expression to the biological features of this malignancy. Our results showed that B7-H3 was highly expressed in the cell line Eca-109 and TE-1, the high expression level of B7-H3 in esophageal cancer tissues was significantly associated with tumor invasion and patient's poor survival. Moreover, the higher B7-H3 expression was significantly and inversely correlated to the $CD3^{+}$T cells infiltration in tumor nest of esophageal cancer tissues. We successfully constructed the recombinant lentivirus of siRNA targeting B7-H3, and the cellular studies showed that the down regulation of B7-H3 expression could suppress the proliferation, colony formation, migration and invasion in Eca-109 cells, which was consistent with the finding from the clinical sample cohort study. Collectively, the high B7-H3 expression was involved in the cancer progression of human esophageal cancer, and might contributed to the negative regulation of T-cell mediated antitumor response in tumor microenvironment, and the proliferation and mobility of esophageal cancer cells. The detailed mechanism and the potential value of clinical use targeting B7-H3 against human esophageal cancer merit further investigation.

Keywords: B7-H3, esophageal cancer, infiltrating $CD3^{+}$T cells, prognosis, RNA interference

Introduction

Esophageal cancer is one of the most common cancer types worldwide, especially with a high incidence in western and northern China, southern and eastern Africa, parts of south America, and Japan [1-3]. Generally, the incidence rates of esophageal cancer are three to four times higher in men than in women, and the mortality rates of esophageal cancer are the fifth and the eighth in men and in women, respectively [4]. The squamous cell carcinoma and the adenocarcinoma are the two main types of human esophageal cancer according to the histopathological classification [5]. The squamous cell carcinoma represents 90% of all esophageal cancers, and especially is the major type of esophageal cancers in China [5, 6]. Despite availability of multiple strategies of diagnosis and therapeutics in recent decades for this malignancy, the postoperative prognosis and the overall 5-year survival rate of the patients still remain very poor [7]. Thus it's important for us to explore the novel biomarkers to benefit the early diagnosis and the targeted treatment strategy to improve patient's postoperative prognosis of esophageal cancer.

It is well known that effective activation of naïve T cells requires two signals, the first signal is

Table 1. Correlation between clinical parameters and B7-H3 expression

Clinical parameters	Cases	B7-H3 immunostaining score			*P*-value
		H-score≤185 (%)	*H-score*>185 (%)	χ^2	
Gender					
Male	128	56 (43.75)	72 (56.25)	0.0496	0.8237
Female	46	21 (45.65)	25 (54.35)		
Age (years)					
<60	100	43 (43.00)	57 (57.00)	0.1496	0.6989
≥60	74	34 (45.95)	40 (54.05)		
Tumor size (cm)					
<3.5	62	30 (48.39)	32 (51.61)	0.6673	0.4140
≥3.5	112	47 (41.96)	65 (58.04)		
Depth of invasion (T)					
T_1	20	15 (75.00)	5 (25.00)	5.411	**0.0200**[a,b]
T_2	67	29 (43.28)	38 (56.72)		
T_3	66	25 (37.88)	41 (62.12)		
T_4	21	8 (38.10)	13 (61.90)		
Nodal metastasis (N)					
Yes	75	31 (41.33)	34 (58.67)	0.4554	0.4998
No	99	46 (46.46)	53 (53.54)		
Distant metastasis (M)					
Yes	21	8 (38.10)	13 (61.90)	0.3671	0.5446
No	153	69 (45.10)	84 (54.90)		
TNM stage					
I	14	9 (64.29)	5 (35.71)	1.675	0.1956[b]
II	102	45 (44.12)	57 (55.88)		
III	37	15 (40.54)	22 (59.46)		
IV	21	8 (38.10)	13 (61.90)		

[a]Values in bold signify $P<0.05$; [b]Chi square test for trend.

presented by the combination of the antigen-specific T cell receptor (TCR) and the major histocompatibility complex (MHC) on antigen-presenting cells (APC), and the second signal is provided by the co-stimulatory molecules binding to their receptors, namely co-stimulatory signals [8, 9]. B7/CD28 family members, an important cohort of co-stimulatory molecules, play an essential role in the T cell mediated immune response [10]. Recently, the bulk of data indicated that some of the B7 family members could also be expressed in cancer cells, and are engaged in oncogenesis and associated with tumor progression [11, 12]. The involvement of co-stimulatory signal confers increasing complexity to the regulation mechanism of T cell mediated anti-tumor immune response, and also provides novel strategies for cancer immunotherapy. Nowadays, the clinical use of the blockade of PD-1/PD-L1 pathway has made a breakthrough in cancer immunotherapy and receives the unprecedented attention [10, 13, 14]. Our previous data demonstrated that high expression levels of B7-H1 and B7-H4 could be found in the upper gastrointestinal cancer tissues in contrast to adjacent normal tissues, and significantly associated with the cancer progression and patients' prognoses [9, 15, 16]. We also showed that another important co-inhibitory molecule B7-H3, was highly expressed in human colorectal cancer tissues, and its expression level was positively correlated with tumor grade, and was negatively correlated with the intensity of tumor infiltrating T lymphocytes, which suggested that B7-H3 was involved in colorectal cancer progression and evasion of cancer immune surveillance [17].

B7-H3, a recently identified important co-stimulatory molecule, is a type I transmembrane protein that shares 20%~27% amino acid identity with other B7 family members [18]. The murine

B7-H3 consists of a single extracellular variable-type IgV-IgC domain and a signature intracellular domain (2 Ig B7-H3), and the human B7-H3 possesses an additional isoform, 4Ig B7-H3, which contains a nearly exact tandem duplication of the IgV-IgC domain [19]. The receptor of B7-H3 still remains unknown, thus the role of B7-H3 in adaptive immune responses still remains elusive [20]. Recent studies demonstrated that the higher B7-H3 expression on tumor cells is associated with poorer outcome of patients suffering from prostate cancer, clear cell renal cell carcinoma, ovarian cancer and hepatocellular carcinoma [21-25]. Moreover, the tumor cell expressed B7-H3 was also suggested to be correlated with the biological behaviors of cancer cells, such as migration, invasion, and metastatic capacity, and finally contributed to cancer progression [26-28].

In the present study, we focused on B7-H3 expression in human esophageal cancer tissues as well as human esophageal cancer cells lines. We found that higher B7-H3 expression was associated with tumor progression, poor survival and $CD3^+$T cell infiltration in human esophageal cancer. Flow cytometry also showed that B7-H3 was highly expressed on human esophageal cancer cells lines Eca-109 and TE-1. The knockdown expression of B7-H3 could inhibit the proliferation, colony formation ability, migration and invasion of Eca-109, suggesting that the B7-H3 expressed by tumor cells could significantly promote cancer progression in this malignancy.

Materials and methods

Patient and tissues samples

Formalin-fixed, paraffin-embedded esophageal cancer tissue samples were collected from 174 patients who underwent surgical resection between December 2000 and May 2006 in our hospital (128 men and 46 women; median age at diagnosis was 58 years). In addition, 6 normal tissues from the non-malignant portion of esophagus were collected from surgery and used as controls. No patients received preoperative chemotherapy or radiotherapy. All tumor tissues were confirmed as the esophageal squamous cell carcinoma by using hematoxylin and eosin (H&E) staining after surgical resection. The detailed clinical parameters of the patients are shown in **Table 1**. The protocols for the present study were approved by the ethics committee of the hospital.

Antibodies and major reagents

Goat anti-human B7-H3 polyclonal antibody and mouse anti-human B7-H3 monoclonal antibody were purchased from R&D Systems (Minneapolis, MN, USA), goat anti-mouse IgG-HRP were purchased from Santa Cruz (Dallas, TX, USA). APC-conjugated mouse anti-human B7-H3 monoclonal antibody and APC-conjugated mouse IgG1 Isotype control were purchased from eBioscience (San Diego, CA, USA). Mouse anti-human CD3 monoclonal antibody (ready to use) was purchased from Maixin Biotechnology (Fuzhou, China). Polink-2 plus® polymer HRP detection system for goat primary antibody (PV-9003) was purchased from Zhongshan Golden Bridge Biology (Beijing, China). An RNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA). SYBR Green Master Mix kits were purchased from TaKaRa (Dalian, China). RPMI-1640, DMEM and fetal bovine serum (FBS) were purchased from Gibco (Cambrex, MD, USA).

Cell lines and cell culture

Human esophageal cancer cell lines Eca-109 and TE-1 were obtained from Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences. The two esophageal cancer cell lines were cultured in RPMI1640 or in DMEM, supplemented with 10% FBS, and were incubated at standard culture conditions (5% CO_2, 37°C).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 3-μm-thick consecutive sections, and were dewaxed in xylene, rehydrated and graded ethanol solutions. Antigens were retrieved by heating the tissue sections at 100°C for 30 min in citrate (10 mmol/L, pH6.0) (for CD3) or EDTA (1 mmol/L, pH9.0) solution (for B7-H3) when needed. Then, the sections were immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in phosphate buffered saline (PBS) for 5 min, blocked with 3% BSA at room temperature for 30 min, and incubated with purified goat anti-human B7-H3 antibody (final concentration in use, 2.5 μg/ml) at 4°C overnight. A negative control was performed by omitting

the primary antibodies. The Polink-2 plus® polymer HRP detection system for goat primary antibody was used according to the manufacture's instruction, and then the diaminobenzene was used as the chromogen, and hematoxylin as the nuclear counterstain. Sections were dehydrated, cleared and mounted.

Evaluation of immunohistochemical staining

All slides were examined independently by two senior pathologists who were not informed of patients' clinical parameters. The B7-H3 immunostaining densities were assessed according to the *H-score* method which has been described by our previous reports [29, 30]: *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% strong staining tumor cells). Results from the two pathologists were averaged and used in the statistical analysis. The infiltrating $CD3^+$T cells the tumor nest of esophageal cancer tissues were determined according to the methods in our previous studies [16, 17, 31]. In brief, The infiltrating $CD3^+$T cells the tumor nest were counted as follows: five areas in tumor nest with the most intense infiltrating $CD3^+$T cells were selected at low magnification (×40), and then the infiltrating $CD3^+$T cells were counted and recorded at high power field (HPF, ×200 magnification). Results from the five areas were averaged and used in the statistical analysis.

B7-H3 RNAi lentivirus generation, infection and cell sorting

The human esophageal cancer cell line Eca-109 was used in the B7-H3 RNAi study. The small hairpin RNA (shRNA) of the human B7-H3 (NM_001024736; GenBank) lentiviral gene transfer vector encoding the green fluorescent protein (GFP) sequence was constructed by Shanghai GeneChem Co. Ltd (Shanghai, China) as described in the previous study [28]. The targeting sequence of B7-H3 was 5'-GAGCAGGGCTTGTTTGATGTG-3', and the recombinant lentivirus of siRNA targeting B7-H3 (LV-B7-H3-siRNA virus) and the non-targeted control mock lentivirus (LV-NC virus) were prepared and transfected to the Eca-109 according to the manufacture's instruction. The infected cells were termed the LV-B7-H3-siRNA group and LV-NC group, respectively, and the un-infected Eca-109 cells were the control group. Then, the infected cells were analyzed by flow cytometry (Cantoll, BD, USA) and sorted by GFP via FL1 channel by flow sorter (Aria II, BD, USA).

Real-time reverse transcriptase-polymerase chain reaction

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to confirm the knockdown of B7-H3 mRNA expression. Total RNA from Eca-109 cells was extracted by using TRIzol (Invitrogen), and was then reverse transcribed into cDNA by using a RT reaction kit (Promega). Real-time PCR was performed by using the ABI 7600 system (Applied Biosystems, USA) according to the manufacturer's instruction and SYBR Green as a DNA-specific fluorescent dye. Primer sequences for detection of the reference gene GAPDH and the target gene B7-H3 were synthesized as follows, the human GAPDH forward primer: 5'-TGACTTCAACAGCGACACCCA, the human GAPDH reverse primer: 5'-CACCCTGTTGCTGTAGCCAAA-3', the human B7-H3 forward primer: 5'-CTCTGCCTTCTCACCTCTTTG-3', and the B7-H3 reverse primer: 5'-CCTTGAGGGAGGAACTTTATC-3'. The real-time PT-PCR products for B7-H3 and GAPDH were also confirmed by using electrophoresis on 1.8% agarose gel containing 0.1% ethidium bromide. Images of the fluorescent bands were captured by use of the Bio-Rad gel documentation system.

Western blot analysis

The protein expression of B7-H3 and GAPDH was determined by western blot analysis. In brief, the whole cell extracts were prepared from 1×10^6 cells in RIPA lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-eoxycholate, 1 mM EDTA and protease inhibitor cocktail). Cells were lysed on ice for 30 min, the cell lysate was collected into microtubes, and samples were centrifuged for 15 min at 12000 rpm at 4°C. Supernatants were collected and the protein concentrations were measured by using the BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of denatured protein were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Then, the membranes were blocked by using 5% non-fat dry milk in TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.05% Tween-20)

for 3 h at room temperature, and then were incubated with primary antibodies overnight at 4°C. Blots were washed and incubated for 1 h with the anti-rabbit secondary antibody and then after three washes with PBST, immunoreactive protein bands were detected by using an Odyssey Scanning system (Li-Cor, Lincoln, NE, USA).

Cell viability assay

The cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China), and was conducted according to the manufacturer's instruction. In brief, the LV-B7-H3-SIRNA group and the LV-NC group Eca-109 cells (2.5×10^4, respectively) were seeded into each well of a 96-well plate and cultured in 100 μl of RPMI-1640 supplemented with 10% FBS. At the indicated time points, medium was exchanged for 100 μl of RPMI-1640 with CCK-8 reagent (10 μl CCK-8 and 90 μl RPMI-1640), and the cells were incubated for 1 h. Absorbance was measured for each well at a wavelength of 450 nm. An increase or decrease in absorbance values at 450 nm in the experimental wells relative to the initial value indicated cell growth or death, respectively. Cell growth was monitored every 24 hrs over 6 days. All experiments were independently repeated at least three times.

Colony formation assay

The Eca-109-LV-NC and Eca-109-LV-B7-H3-siRNA cells were used in the colony formation assay. In brief, 200 Eca-109 cells were plated in a 6-well plate in complete medium, and the plate was shaken to disperse the cells equally. After incubation at 37°C with 5% CO_2 for 10-12 days, when the colonies were visible by eye, the culture was terminated by removing the medium and washing cells twice with phosphate-buffered saline (PBS). The colonies were fixed with 95% ethanol for 10 min, dried and stained with 0.1% Crystal Violet solution for 10 min, and the plate was washed three times with water. Images were taken of the stained plates, and the numbers of colonies containing more than 50 cells were counted. Each treatment was performed in triplicate.

Wound healing assay

Cell migration was evaluated by the wound scrape assay to determine whether B7-H3 could be involved in the regulation of the migration of esophageal cancer cells. In brief, the LV-B7-H3-SIRNA group and the LV-NC group Eca-109 cells were incubated in 6-well plates respectively. A small wound area was made in the 90% confluent monolayer by using a 200 μl pipette tip in a lengthwise stripe. Cells were then washed twice with PBS and incubated in serum-free RPMI-1640 medium at 37°C in a 5% CO_2 incubator for 24 hours. Photographs were taken at different time points from 0 to 24 hours. Wound width was measured at 100× magnification by using a B×50 microscope (Olympus®) with a calibrated eyepiece grid (1 mm/20 μm graduation). Ten measurements were made at random intervals along the wound length. Data were averaged and expressed as a percent of the original width. This experiment was done in triplicate.

Transwell invasion assay

The co-culture system was used to evaluate the regulation of invasiveness in Eca-109 cells as described in the previous study [27]. In brief, the upper portion of Transwell® inserts with an 8 μm pore size and a 6.5 mm diameter was coated with 20 μl matrigel diluted 1:3 in serum-free RPMI-1640 and incubated at 37°C for 4 hours. The coated inserts were placed in the well of 24-well plate with 600 μl RPMI-1640 medium containing 10% FBS in the bottom chamber. After 12 hours of serum starvation the trypsinized LV-B7-H3-siRNA group and LV-NC group Eca-109 cells respectively, were harvested and diluted to a 1×10^6/ml cell suspension with serum-free RPMI-1640 medium. Each cell suspension (100 μl) was added to the upper chambers. After incubation at 37°C for 24 hours in a 5% CO_2 atmosphere the non-invading cells and gel were removed from the upper chamber with cotton tipped swabs. The cells were rinsed with PBS and cells on the filters were fixed with methanol for 30 minutes and stained with crystal violet solution (Sigma). The number of invading cells on the filters was counted in 5 random fields per filter at 100× magnification in triplicate wells of each group.

Statistical analyses

The paired Student's *t*-test, the Wilcoxon signed rank test, the Chi-square test or the Log-rank survival analysis were used where appropriate. All the statistical analyses were performed by using the GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). A p-value of <0.05 was deemed significant.

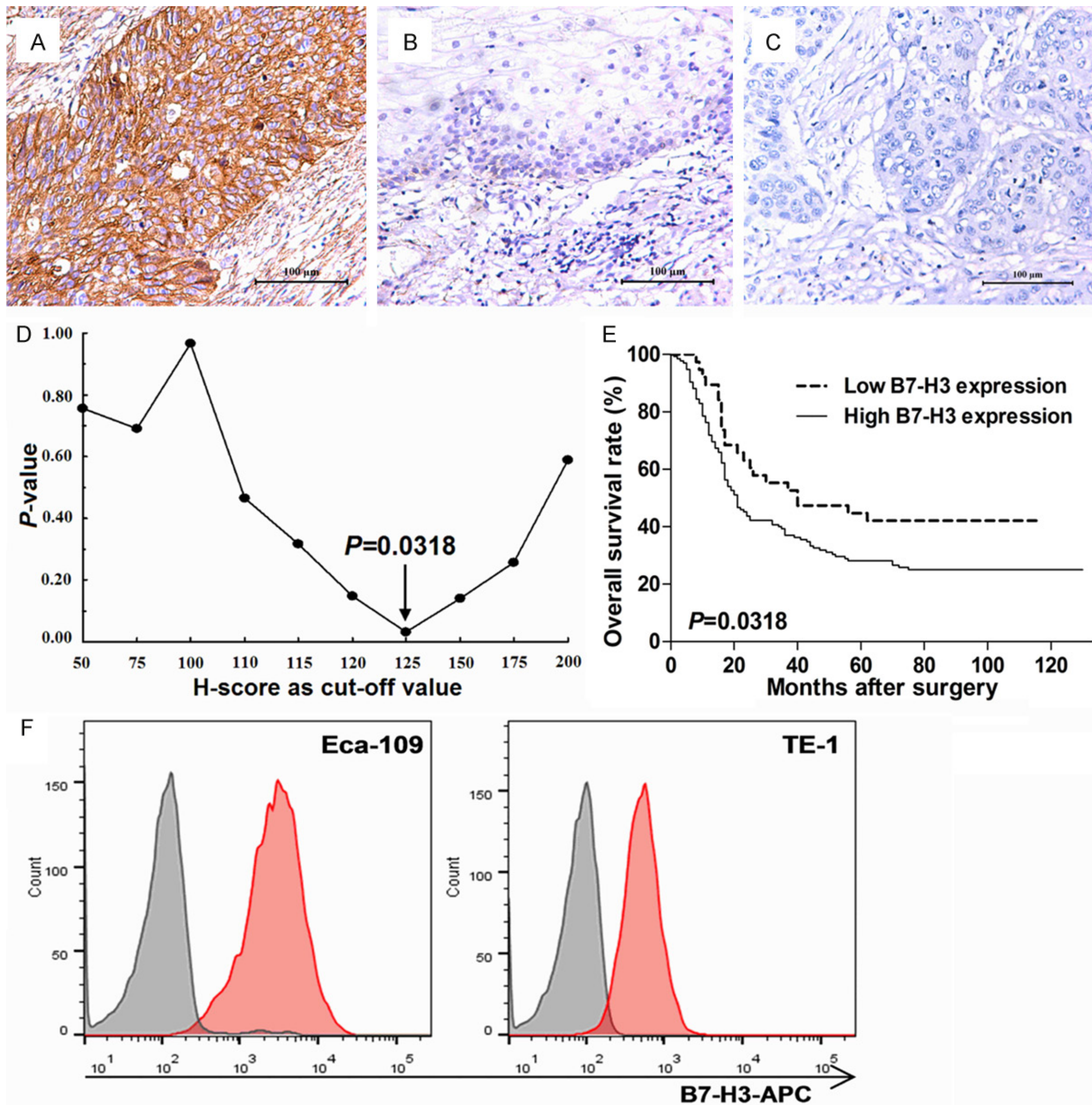


Figure 1. B7-H3 expression in human esophageal cancer tissues and cell lines. A. B7-H3 expression could be found in human esophageal cancer tissues, and positive staining of B7-H3 was mainly located on the membrane of cancer cells. B. Weak and negative staining of B7-H3 could be found in normal esophageal tissues. C. Negative control (Scale bar=100 μm in panel A to C). D. The minimum *P*-value seek was conducted according to the method as described in the literatures and our previous study by using different *H-score* values as cut-offs [29, 30, 32]. E. When we selected *H-score*=125 as cut-off value, the survival analysis demonstrates that the overall survival rate of the subgroup with lower B7-H3 expression (0≤*H-score*≤125) is significantly better than that the subgroup with higher B7-H3 expression (*H-score*>125) (*P*=0.0318, Hazard Ratio: 1.564, 95% CI, 1.040-2.351). F. Flow analysis showed B7-H3 could be expressed in the human esophageal cancer cell lines Eca-109 and TE-1, the red shadings indicate specific membranous staining of B7-H3, and the black shadings indicate Isotype controls.

Results

B7-H3 expression in human esophageal cancer tissues and cancer cell lines

The results from the immunohistochemistry assay showed that the positive B7-H3 immunohistochemical staining was predominantly observed on the membrane and in cytoplasm of esophageal cancer cells (**Figure 1A**), while none or weak staining of B7-H3 was found in adjacent normal esophageal tissues (**Figure 1B**). In order to further investigate the correlation between clinical parameters and the B7-H3

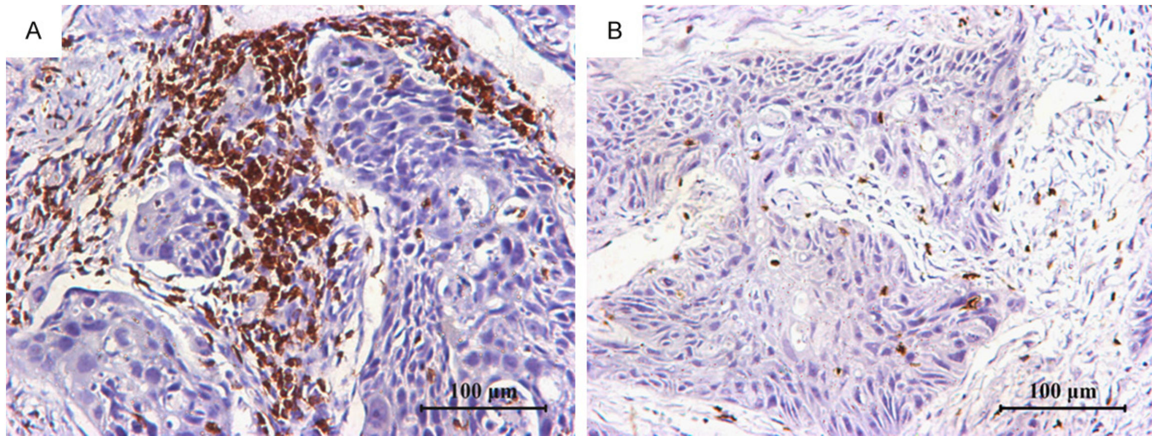


Figure 2. Infiltrating $CD3^+$T cells in esophageal cancer tissues. A. High intensity of infiltrating $CD3^+$T cells in esophageal cancer tissue; B. Low intensity of infiltrating $CD3^+$T cells in esophageal cancer tissue. (Scale bar=100 μm).

Table 2. Correlation between the intensity of infiltrating $CD3^+$T cells and B7-H3 expression in esophageal cancer tissues

Intensity of infiltrating $CD3^+$T cells	Cases	B7-H3 expression level				X^2 value	*P* value
		Low (*H-score*≤185)	Ratios (%)	High (*H-score*>185)	Ratios (%)		
Low (≤60/HPF)	62	13	20.97	49	79.03	5.022	**0.0250**
High (>60/HPF)	41	17	41.46	24	58.54		

Bold signifies $P<0.05$.

immunostaining intensity in the esophageal cancer tissues, we categorized 174 patients into two major subgroups according to the intensity (*H-score*) of B7-H3 immunohistochemical staining, i.e., the lower B7-H3 expression group, 77 cases (0≤*H-score*≤185), and the higher B7-H3 expression group, 97 cases (*H-score*>185). Our flow cytometry analysis also showed that B7-H3 could be expressed on the membrane of the human cancer cell lines, Eca-109 and TE-1 respectively (**Figure 1F**).

B7-H3 expression in relation to patient's clinical parameters and postoperative prognosis

The correlation between patient's clinical parameters and tumor cell B7-H3 expression is shown in **Table 1**. Our data showed that the B7-H3 expression in human esophageal cancer tissues is significantly associated with tumor invasion depth (P=0.0200), whereas it is not correlated with patient's gender, age, tumor size, nodal metastasis, distant metastasis and TNM stage. Thus, our data suggests that higher B7-H3 expression of esophageal cancer tumor cells protein is positively correlated with tumor invasion, suggesting that B7-H3 expression is involved in the progression of this malignancy. Moreover, B7-H3 expression in relation to patient's postoperative prognosis was examined by log-rank survival analysis, in which the minimum *P*-value seek was conducted according to the method as described in the literatures and our previous study by using different *H-score* values as cut-offs [29, 30, 32], and when we selected *H-score*=125 as the cut-off value, the survival analysis demonstrates that the overall survival rate of the subgroup with lower B7-H3 expression (0≤*H-score*≤125) is significantly better than that the subgroup with higher B7-H3 expression (*H-score*>125) (P=0.0318, Hazard Ratio: 1.564, 95% CI, 1.040-2.351, **Figure 1D** and **1E**).

B7-H3 expression in relation to infiltrating $CD3^+$T cells in cancer tissues

We also examined the number of infiltrating $CD3^+$T cells in tumor nest of esophageal cancer tissues by using immunohistochemical staining of CD3 marker (**Figure 2A** and **2B**), and then evaluated the correlation of the intensity of infiltrating $CD3^+$T cells in tumor nest and B7-H3 expression level in specimens from 103 patients from all the 174 cases. We selected the cut-off values of 60/HPF for of infiltrating $CD3^+$T cells in tumor nest to divided the intensity of $CD3^+$T cell in cancer tissues, namely 62 cases

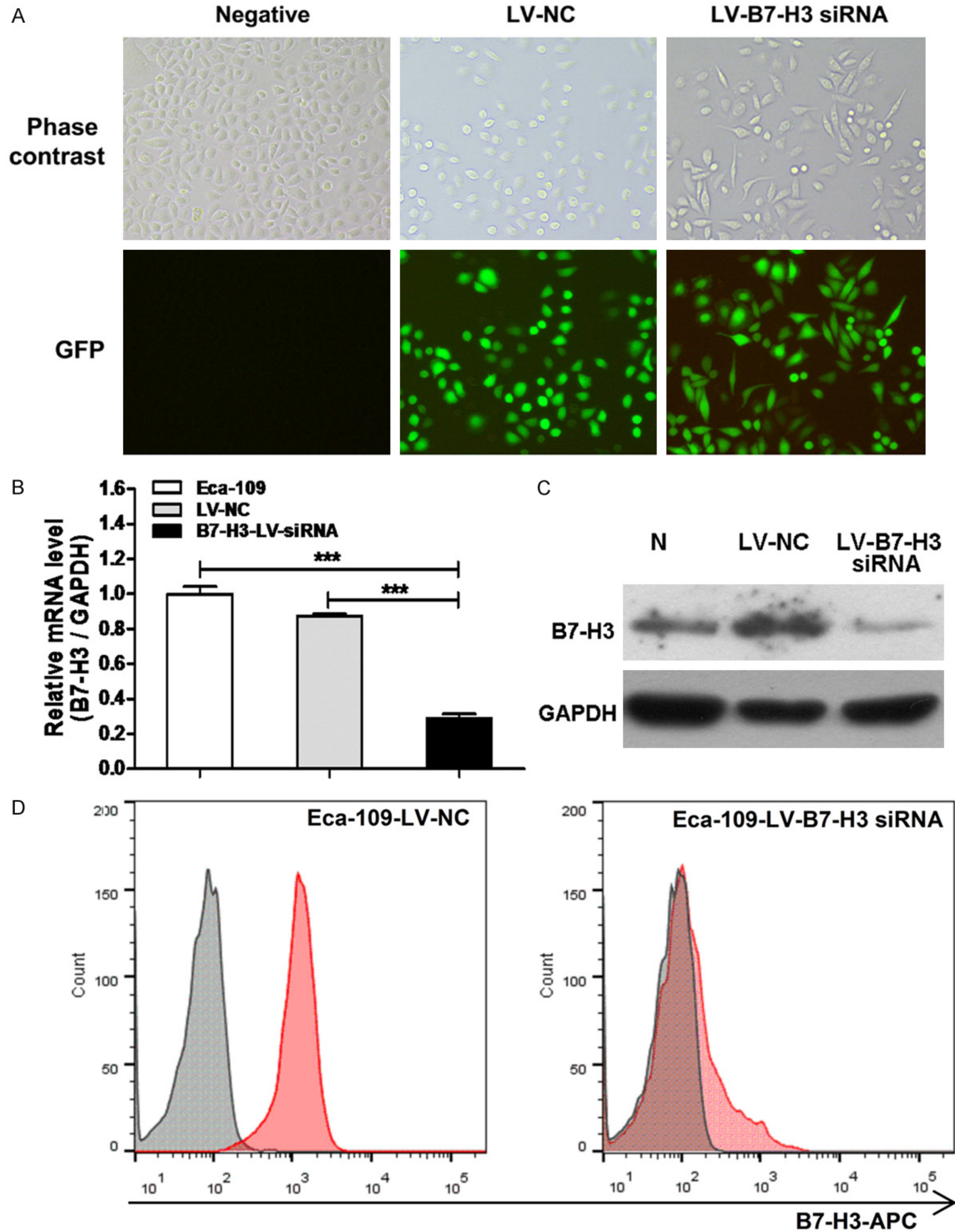


Figure 3. Down regulation of B7-H3 expression in human esophageal cancer cell line Eca-109. A. The efficiency of infection was confirmed by detecting GFP expression by using fluorescence microscopy. B. The down regulation of B7-H3 mRNA expression after knock-down by RNAi method in Eca-109 cells was verified by using real-time RT-PCR, which showed that the B7-H3 mRNA expression in LV-B7-H3-siRNA group was significantly lower than that in LV-NC group and Eca-109 control group, respectively (both $P<0.001$). C. The down regulation of B7-H3 protein expression after knock-down by RNAi method in Eca-109 cells was verified by using western blot method, which showed that the B7-H3 protein expression was decreased in LV-B7-H3-siRNA group in contrast to the LV-NC group and Eca-109 control group, respectively. D. The down regulation of B7-H3 protein expression after knock-down by RNAi method in Eca-109 cells was also verified by using flow analysis, which showed that the B7-H3 protein expression was de-

creased in LV-B7-H3-siRNA group in contrast to the LV-NC group, the red shadings indicate specific membranous staining of B7-H3, and the black shadings indicate Isotype controls.

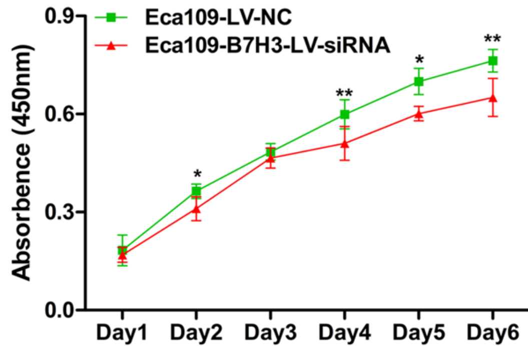


Figure 4. Down-regulation of B7-H3 expression suppresses the proliferation of Eca-109 cells. We examined the down regulation of B7-H3 on the cell proliferation rate *in vitro* by using CCK-8 assay in Eca-109-LV-B7-H3-siRNA and Eca-109-LV-NC groups. On day 2, 4, 5 and day 6, the proliferation rate of LV-B7-H3-siRNA group was significantly lower than that of LV-NC control group, $P<0.05$, $P<0.005$, $P<0.05$, and $P<0.005$, respectively.

with low intensity of infiltrating $CD3^+$T cells, and 41 cases with high intensity of infiltrating $CD3^+$T cells. As shown in **Table 2**, we found that B7-H3 expression level in human esophageal cancer tissues was significantly and inversely correlated with the intensity of infiltrating $CD3^+$T cells ($\chi^2=5.022$, $P=0.0250$), suggesting that the co-stimulatory molecule B7-H3 might be involved in the suppression of T-cell mediated anti-tumor response.

Down regulation of B7-H3 expression in esophageal cancer cell line Eca-109

In order to further investigate the regulation of B7-H3 on the biological features of esophageal cancer cells, we firstly constructed the recombinant lentivirus of siRNA targeting B7-H3 (LV-B7-H3-siRNA virus) and the non-targeted control mock lentivirus (LV-NC virus), and then transfected them to the human esophageal cancer cell line Eca-109. Furthermore, we sorted LV-B7-H3-siRNA virus transfected Eca-109 cells (Eca-109-LV-B7-H3-siRNA) or LV-NC virus transfected Eca-109 cells (Eca-109-LV-NC) by using by Aria II flow sorter (BD, USA) based on GFP via FL1 channel. The efficiency of infection was also confirmed by detecting GFP expression by using fluorescence microscopy (**Figrue 3A**). Then, we used real-time RT-PCR to further confirm the B7-H3 gene expression level in Eca-109-LV-B7-H3-siRNA, Eca-109-LV-NC and Eca-109 cells as control, and the results showed that the B7-H3 mRNA expression in LV-B7-H3-siRNA group was significantly decreased in contrast to LV-NC as well as The control group, respectively ($P<0.001$, **Figrue 3B**). The western blot analysis and the flow analysis also showed that the B7-H3 protein expression in the LV-B7-H3-siRNA group was significantly decreased in contrast to the LV-NC group, respectively (**Figrue 3C** and **3D**).

Down-regulation of B7-H3 expression suppresses the proliferation and the colony formation ability of esophageal cancer cells

In order to verify the role of B7-H3 in the growth of human esophageal cancer cells, we next examine the down-regulation of B7-H3 expression on the cell proliferation rate *in vitro* by using CCK-8 assay in Eca-109-LV-B7-H3-siRNA and Eca-109-LV-NC groups. As shown in **Figure 4**, we found that both on day 2, 4, 5 and day 6, the proliferation rate of LV-B7-H3-siRNA group was significantly lower than that of LV-NC control group, $P<0.05$, $P<0.005$, $P<0.05$, and $P<0.005$, respectively, which suggests that high B7-H3 expression in esophageal cancer cells might role importantly in promoting cell proliferation and cancer progression in human esophageal cancer. As shown in **Figure 5**, by using the colony formation assay, we also found the clone number of LV-NC group was significantly higher than that of LV-B7-H3-siRNA group, which suggests that B7-H3 molecule expressed by esophageal cancer cells might role importantly in regulating the colony formation ability.

Down-regulation of B7-H3 expression inhibits migration and invasion of esophageal cancer cells

In order to study whether the down-regulation of B7-H3 affects the cellular migration and invasion, we further carried out wound healing assay and transwell assay on the esophageal cancer cells in LV-B7-H3-siRNA group as well as LV-NC group respectively. The wound healing assay results showed that the migration ability of the LV-B7-H3-siRNA group cells was significantly lower than that in LV-NC group. As shown in **Figure 6**, the cell-free area of the LV-B7-H3-siRNA group was significantly wider than that of

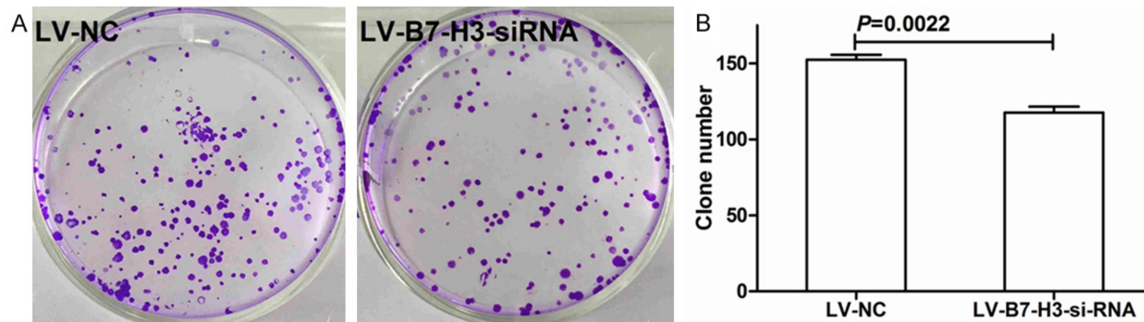


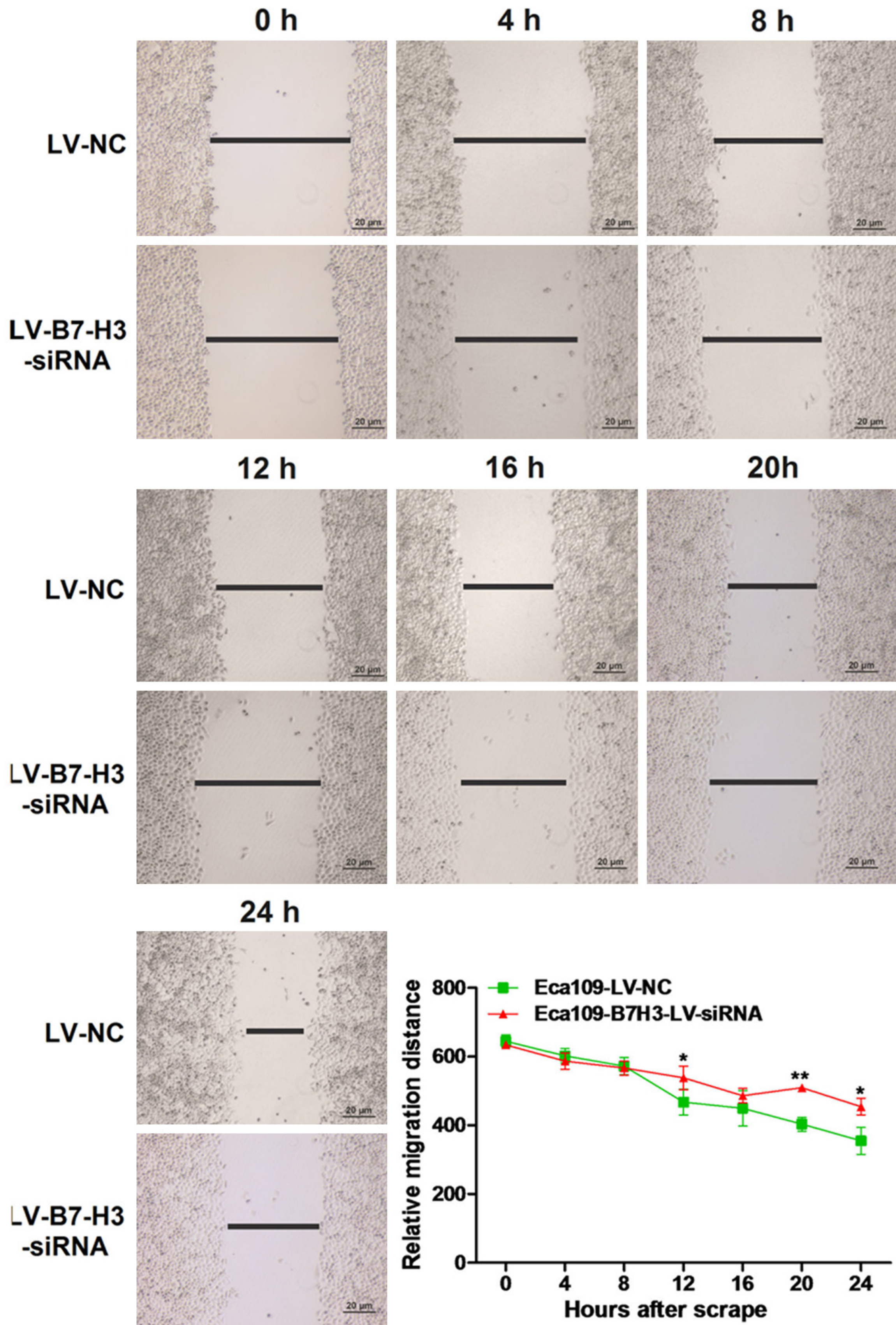
Figure 5. Down-regulation of B7-H3 expression inhibits the colony formation ability of Eca-109 cells. A. Colony formation assays showed Crystal Violet staining of the NC-transfected group and B7-H3 siRNA-transfected group in Eac-109 cells. B. Graph indicates colony numbers of each experimental transfected groups ($P<0.005$).

LV-NC group at 12 h ($P<0.05$), 20 h ($P<0.005$) and 24 h ($P<0.05$), respectively, after drawing the scratch line on the monolayer cells, which suggested that the down-regulation of B7-H3 expression could significantly decrease the cell migration of Eca-109 cells in the wound scrape model. In the transwell invasion assay, the number of invaded cells stained with Cristal Violet was significantly less in the LV-B7-H3-siRNA group (**Figure 7A**). Invasion rates of LV-B7-H3-siRNA group as well as LV-NC group Eca-109 cancer cells were also detected by counting the number of cells that invaded through matrigel and confirmed the results observed by inverted microscope (**Figure 7B**). Based on these results, we demonstrated here that the down-regulation of B7-H3 expression could suppress cellular invasion in human esophageal cancer cells, which confirmed our finding from the clinical sample cohort study that the higher B7-H3 expression in human esophageal cancer tissues was significantly and positively associated with tumor invasion depth.

Discussion

It has been demonstrated that many members from B7 family ligands not only role importantly in the regulation of T-cell mediated immune response, but also have essential implications in human cancer progression [9]. Some inhibitory B7 family ligands, such as B7-H1, B7-H3 and B7-H4, are highly expressed in various human cancers, and their expression levels significantly associated with cancer progression and patients' prognoses [11, 12, 33]. We have previously examined B7-H4 protein expression as well as densities of various tumor infiltrating lymphocyte (TILs) subsets in human esophageal cancer tissues by using immunohistochemistry, and demonstrated that B7-H4 expression in human esophageal cancer was significantly correlated with distant metastasis, cancer progression, densities of TILs, and poorer patient outcome [16]. In addition, we also found that B7-H1 expression could be found in human esophageal cancer cell lines and tissues, and higher B7-H1 expression in human esophageal cancer tissues was significantly correlated with tumor invasion depth and poor prognosis [33]. As of now, the clinical significance of B7-H3 expression in human esophageal cancer and its biological function in cancer progression of this malignancy still remain elusive.

In the present study, we firstly retrospectively studied the clinical implications in human esophageal cancer in a cohort of 174 cancer patients. Our results showed that higher B7-H3 expression in human esophageal cancer tissues was significantly associated with tumor invasion and poor prognosis of the patients. The clinical significance of B7-H3 in some human cancers has been concerned. Our previous study showed that higher B7-H3 expression in human colorectal cancer tissues was also positively and significantly associated with tumor stage [17]. B7-H3 was also highly expressed by prostate cancer cells, correlated with Gleason score, seminal vesicle involvement, surgical margin, extra-prostatic extension and poor prognosis [21]. Sun and his colleagues also showed that B7-H3 was expressed in human hepatocellular carcinoma and was associated with tumor aggressiveness and postoperative recurrence [25]. It has been demonstrated that the expression of B7-H3 in hypopharyngeal squamous cell carcinoma could be used as a predictive indicator for tumor metastasis and prognosis [34]. Thus, combined with our retrospective study results and others', the B7-H3 could be used as a valuable prognos-



0 h
4 h
8 h
LV-NC
LV-B7-H3
-siRNA
12 h
16 h
20h
LV-NC
LV-B7-H3
-siRNA
24 h
LV-NC
LV-B7-H3
-siRNA
20 μm
Eca109-LV-NC
Eca109-B7H3-LV-siRNA
Relative migration distance
Hours after scrape
800
600
400
200
0
0
4
8
12
16
20
24
*
**
*

Figure 6. Down-regulation of B7-H3 expression inhibits the migration of Eca-109 cells. The wound healing assay on Eca-109 cells in LV-B7-H3-siRNA group as well as LV-NC group showed that, the cell-free area of the LV-B7-H3-siRNA group was significantly wider than that of LV-NC group at 12 h ($P<0.05$), 20 h ($P<0.005$) and 24 h ($P<0.05$) respectively after drawing the scratch line on the monolayer cells.

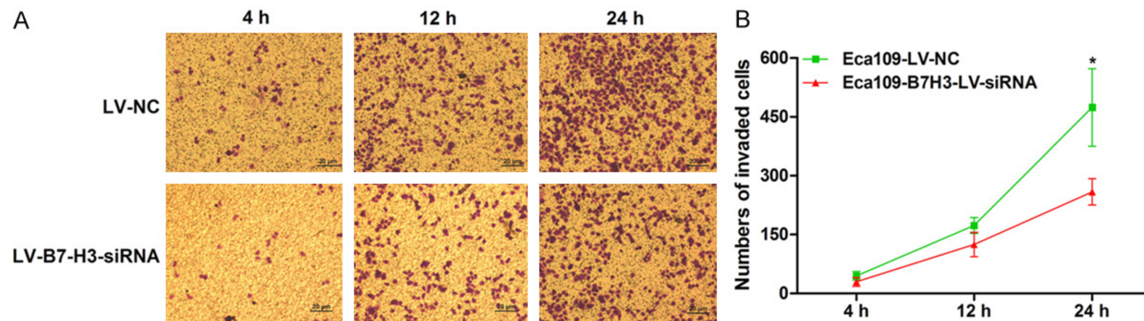


Figure 7. Down-regulation of B7-H3 expression inhibits the invasion of Eca-109 cells. A. The transwell invasion assay showed that the number of invaded cells stained with Cristal Violet was significantly less in the LV-B7-H3-siRNA group in contrast to the LV-NC-siRNA group. B. The invasion rate was detected by counting the number of cells that invaded through matrigel and confirmed the results observed by inverted microscope, which showed that at the time point of 24 h, the number of invaded cells in the LV-B7-H3-siRNA group was significantly lower than that in the LV-NC group ($P<0.05$).

tic predictor and a potential therapeutic target in human esophageal cancer.

The presence of tumor infiltrating T cells has been used as an indicator for tumor immune surveillance in human colorectal cancer [35]. Our previous study also showed that higher B7-H3 expression in human colorectal cancer tissues was significantly and inversely associated with the density of tumor infiltrating $CD3^+$T cells, suggesting the B7-H3 may play a suppressive role in tumor microenvironment via regulating the T-cell mediated anti-tumor immune response [17]. It has been reported that B7-H3 molecule could inhibit T-cell activation and production of interferon-γ through regulation of NFTA, NF-κB and AP-1 [25, 36]. The B7-H3 expressed by the cancer cells could also inhibit the proliferation of $CD8^+$T cells in human hepatocellular carcinoma [37]. Thus, combined with our finding that the B7-H3 expression level in human esophageal cancer significantly and inversely associated with the density of tumor infiltrating $CD3^+$T cells, we supposed that the negative immune-regulation role of B7-H3 in the cancer progression.

Numerous studies recently showed that aberrant tumor cell expressed B7-H3 could also associated with the biological features of tumor cells themselves. Zhao and his colleagues [28] reported that down-regulation of B7-H3 expression in human pancreatic cancer cells could inhibit the proliferation, the migration and the invasion of cancer cells. It also has been shown that down-regulation of B7-H3 expression in human prostate cancer cells could decrease cell adhesion, migration and invasion [27]. B7-H3 was over-expressed in human hepatocellular carcinoma, and higher expression of B7-H3 in cancer cells could promote cell proliferation, adhesion, migration and invasion [37]. In our present study, we also found that B7-H3 was highly expressed on esophageal cancer cells as well as esophageal cancer tissues, thus we further aimed to study the potential role of B7-H3 in regulation of the biological functions of esophageal cancer cells.

We then successfully constructed the model of down-regulation of B7-H3 expression in esophageal cancer cell Eca-109 by using siRNA method and further studied its implication to cell biological functions. We firstly examined the down-regulation of B7-H3 on the cell proliferation rate in vitro by using CCK-8 assay, and we found that the proliferation rate of LV-B7-H3-siRNA group was significantly lower than that of LV-NC control group. By using the colony formation assay, we also found the clone number of LV-NC group was significantly higher than that of LV-B7-H3-siRNA group, which suggested that the B7-H3 molecule expressed by esophageal cancer cells might role importantly in regulating

the colony formation ability. Thus, these data suggested that B7-H3 expression in esophageal cancer cells might role importantly in promoting cell proliferation and cancer progression in human esophageal cancer. Moreover, we also found that down-regulation of B7-H3 expression could inhibit migration and invasion of esophageal cancer cells by using wound healing assay and transwell invasion assay, and showed that the B7-H3 molecule expressed by cancer cells could promote the mobility of the cancer cells themselves.

In summary, our present study indicated that the co-stimulatory molecule B7-H3 is involved in human esophageal cancer progression. Higher B7-H3 expression in esophageal cancer tissues was significantly associated with tumor invasion, poor prognosis and decreased infiltrating CD3$^+$T cells. The down-regulation of B7-H3 expression in human esophageal cancer cell lines also showed that B7-H3 was involved in the proliferation, migration and invasion of cancer cells, suggesting that B7-H3 could be a useful predictor for tumor progression and postoperative prognosis, and more efforts were needed for developing immunotherapeutic approaches targeting B7-H3 in this malignancy.

Acknowledgments

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Disclosure of conflict of interest

None.

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