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Original Article

B7-H1 expression associates with tumor invasion and predicts patient's survival in human esophageal cancer

Lujun Chen[1], Haifeng Deng[1], Mingyang Lu[1], Bin Xu[1], Qi Wang[1], Jingting Jiang[1], Changping Wu[1,2]

Departments of [1]Tumor Biological Treatment, [2]Oncology, The Third Affiliated Hospital, Soochow University, Changzhou 213003, Jiangsu, China

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Abstract: B7-H1, an important member of the B7-CD28 super family, has been reported to play an important role in regulation of T-cell mediated anti-tumor response, and also has effect in the biological characteristics of the tumor cells themselves. The bulk of data indicate that cancer immunotherapy targeting the molecule B7-H1 recently has sparked growing interest. We have previously reported that higher expression of B7-H1 in human gastric cancer significantly associated with tumor size, invasion, nodal metastasis, survival and the density of infiltrating $Foxp3^+$ Tregs. In the present study, we used tissue microarray to further study B7-H1 expression in human esophageal cancer tissues and its clinical significance. We found that positive membranous B7-H1 expression could be found in some human esophageal cancer cell lines, and both membranous/cytoplasm and nuclear staining of B7-H1 could be found in esophageal cancer tissues. We demonstrated that the membranous/cytoplasm B7-H1 expression in human esophageal cancer tissues was significantly correlated with tumor invasion depth ($P = 0.0261$), whereas it was not correlated with patient's gender, age, tumor size, nodal metastasis, distant metastasis and TNM stage. The survival analysis showed that the overall survival of the patients with positive B7-H1 membrane/cytoplasm expression was significantly poorer than that of the patients with negative B7-H1 membrane/cytoplasm expression (Hazard ratio = 2.157, 95% CI: 1.017-4.577, $P = 0.0452$). Moreover, we also found that the nuclear B7-H1 expression in human esophageal cancer tissues was significantly correlated with tumor invasion depth ($P = 0.0331$), whereas it was not correlated with other parameters. The log-rank survival analysis showed that there was no statistically significant difference in prognosis between the patients with positive nuclear B7-H1 staining and the patients with negative nuclear B7-H1 staining ($P = 0.6755$). Thus, our data showed that B7-H1 can serve as a prognostic predictor for human esophageal cancer, and also could be an important therapeutic target for the immune therapy against this malignancy.

Keywords: B7-H1, esophageal cancer, tissue microarray, survival

Introduction

The esophageal cancer is one of the most common types of human cancer in the world, especially with a high incidence in southern and eastern Africa, western and northern China, parts of South America, and Japan [1-3]. The incidence rates of esophageal cancer are three to four times higher in men than women, and the mortality rates of esophageal cancer are the fifth and eighth in men and women, respectively [4]. According to the histological classification, the squamous cell carcinoma and the adenocarcinoma are the two main types of human esophageal cancer [5], and the squamous cell carcinoma represents 90% of all esophageal cancer cases worldwide. As of now, the overall 5-year survival rate of esophageal cancer still remains very poor, due to its malignant characteristics and the majority of patients are with advanced stages at the time of diagnosis.

As we know, the activation of T-cell not only needs the signal presented by TCR-MHC, but also the signal delivered by co-stimulatory molecule, such as CD28/B7-1, CD28/B7-2 and PD-1/B7-H1, *etc.* B7-H1 (also named PD-L1 or CD274), is an important member of the B7 family, which can interact with its receptor, PD-1 (programmed death-1, a CD28 family member), inhibit T-cell activation, maintain the exhaustion of T cell, impairing cytokine production, and induce the apoptosis of effector T cells [6, 7].

Table 1. Correlation between clinical parameters and B7-H1 expression on membrane and cytoplasm of cancer cells

Clinical parameters	Cases	B7-H1 immunostaining score			*P*-value
		H-score = 0	H-score > 0	χ^2	
Gender					
Male	76	14	62	0.3590	0.5491
Female	23	3	20		
Age (years)					
< 60	51	10	41	0.4389	0.5077
≥ 60	48	7	41		
Tumor size (cm)					
< 3.5	35	7	28	0.3045	0.5811
≥ 3.5	64	10	54		
Tumor invasion depth (T)					
$T_1 + T_2$	35	10	25	4.947	**0.0261**
$T_3 + T_4$	64	7	57		
Nodal metastasis (N)					
Yes	34	6	28	0.0082	0.9277
No	65	11	54		
Distant metastasis (M)					
Yes	6	0	6	1.324	0.2498
No	93	17	76		
TNM stage					
I	5	3	2	0.9872	0.3204
II	57	7	50		
III	31	7	24		
IV	6	0	6		

Values in bold signify $P < 0.05$.

Previous studies have shown that, B7-H1 is broadly distributed in various tissues and cell types, including T cells, B cells, dendritic cells, nature killer cells, activated vascular endothelial cells, mesenchymal stem cells and cultured bone marrow-derived mast cells, even at the human maternal-fetal interface, which may provide protection the fetus against the maternal immune system [8]. In addition, B7-H1 expression has also been found to be up-regulated in many human solid tumors, including liver, ovary, colorectal, lung, pancreatic, gastric, kidney, breast cancers, and *etc.* [9]. And the cancer cell expressed B7-H1 could provide direct tumor protection, inhibit T cell mediated anti-tumor immunity, and finally lead to cancer immune tolerance and escape [10, 11]. Our previous study showed that positive staining of B7-H1 could be found in 42.2% gastric cancer tissues, and B7-H1 expression level was significantly associated with tumor size, invasion, nodal metastasis, and overall survival [12]. And we further confirmed that B7-H1 expression in human gastric cancer tissues significantly associated with the infiltrating density of $Foxp3^+$ Tregs, and the expression level of another inhibitory co-stimulatory molecule B7-H4 [13].

Ohigashi *et al.* [14] reported that B7-H1 expression could be found in esophageal cancer tissues by using immunohistochemistry in frozen section of this malignancy, and revealed that higher expression of B7-H1 predicted poorer survival of esophageal cancer patients. In the present study, we further studied B7-H1 expression in human esophageal cancer cell lines, and also used tissue microarray to investigate the B7-H1 expression pattern in human esophageal cancer tissues as well as cancer cell lines, and to analyze its clinical significance.

Materials and methods

Patients and tissue microarray

Formalin-fixed, paraffin-embedded esophageal cancer tissue samples were collected from 99 patients who underwent surgical resection between February 2005 and May 2006 in our hospital (76 men and 23 women; median age at diagnosis was 59 years). In addition, 4 normal tissues from the non-malignant portion of esophagus were resected from surgery and used as controls. No patients received pre-operative 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 clinical parameters of the patients are shown in **Table 1**. Among all the patients, the survival data of 56 patients were available. Then, the paraffin blocks of 99 cases of esophageal cancer tissues were used in the construction of tissue microarray. In brief, the H&E-stained standard slides were reviewed from each section of esophageal cancer tissues, and a representative tumor region and the corresponding formalin-fixed paraffin-embedded tissue block were selected for use in the tissue microarray. The viable invasive carcinoma tissue (epithelial cells) and surrounding tumor

stroma from central parts within the tumors were carefully selected and marked on the H&E slides, and then were sampled for the tissue microarray block which was assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA).

Antibodies and major regents

Rabbit anti-human B7-H1 monoclonal antibody (NBP1-03220) was purchased from Novus Biologicals (Littleton, CO, USA). PE-conjugated mouse anti-human B7-H1 monoclonal antibody was purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA). PE-conjugated mouse IgG1 Isotype control was purchased from R&D Systems Inc. (Minnneapolis, USA). The horseradish peroxidase (HRP)-labeled goat anti-mouse/rabbit secondary antibody used in immunohistochemistry was purchased from Dako (Glostrup, Denmark). The cell culture medium and supplements were purchased from HyClone (Thermo, Waltham, USA).

Cell lines and cell culture

Human esophageal cancer cell lines TE-1, Eca-109 and Eca-9706 were obtained from Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences. TE-1 and Eca-9706 were cultured in DMEM, and Eca-109 was cultured in RPMI1640, respectively, and supplemented with 10% FBS. The cell lines were incubated at standard culture conditions (5% CO_2, 37°C).

Flow cytometry analysis of B7-H1 expression in esophageal cancer cell lines

Esophageal cancer cell lines TE-1, Eca-109 and Eca-9706 were examined for membranous B7-H1 expression by using flow cytometry analysis. The cells of those three cell lines were collected after cultivation, and were incubated with PE-labeled mouse anti-human B7-H1 monoclonal antibody for 30 min at room temperature, then washed twice in PBS, and then analyzed by the BD FACSCanto II flow cytometry (BD Biosciences, San Jose, CA, USA).

Immunohistochemistry

The paraffin-embedded esophageal cancer tissue microarray block was cut into 3-μm-thick section. A standard immunohistochemical technique was performed using a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, USA) with the B7-H1 antibody (NBP1-03220, Novus) at a dilution in 1:200. Heat epitope retrieval provided by the immunostainer was done for 30 min.

Evaluation of immunohistochemical staining

All slides were examined independently by two senior pathologists who were not informed of patients' clinical parameters. First, the membranous and cytoplasm B7-H1 immunostaining densities were assessed according to the *H-score* method described by our previous reports [15, 16]: H-score = (% tumor cells unstained x0) + (% tumor cells stained weak x1) + (% tumor cells stained moderate x2) + (% tumor cells stained strong x3). The *H-score*s 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. Second, the nuclear staining of B7-H1 was considered as positive if there is any B7-H1 staining in the nucleus of esophageal cancer cells.

Statistical analyses

Statistical analysis was performed using the GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). 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 deemed significant.

Results

B7-H1 expression in human esophageal cancer cell lines and tissues

As shown in **Figure 1**, we demonstrated that positive membrane B7-H1 expression could be found in human esophageal cancer cell lines TE-1 and Eca-109, while it was weakly expressed on esophageal cancer cell line Eca-9706. By using the automatic immunohistochemical staining system Benchmarker XT, we found 82 cases in all 99 patients representing positive membranous/cytoplasm B7-H1 staining (**Figure 2A**), and 79 cases in all 99 patients representing positive nuclear B7-H1 staining (red arrow, **Figure 3A**).

B7-H1 expression in relation to patient's clinical parameters and survival

The correlation between patients' clinical parameters and B7-H1 expression on membrane and cytoplasm of cancer cells is shown in

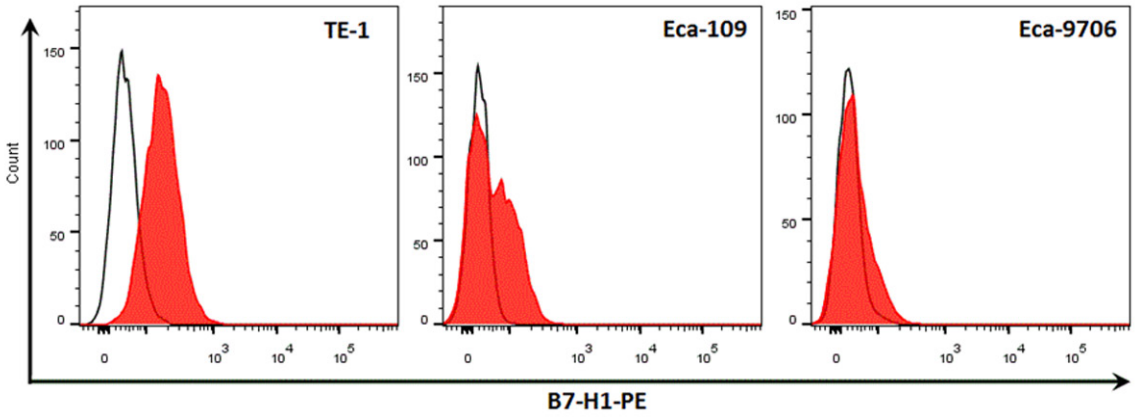

Figure 1. Flow analysis of membrane B7-H1 expression in human esophageal cancer cell lines. We analyze membranous B7-H1 expression in human esophageal cancer cell line by using fluorescence immuno-staining and flow analysis, and we found that membranous B7-H1 expression could be found in TE-1 and Eca-109, while it's was very weakly expressed on Eca-9706. The continuous lines indicate Isotype controls, and the red shadings indicate specific membranous staining of B7-H1.

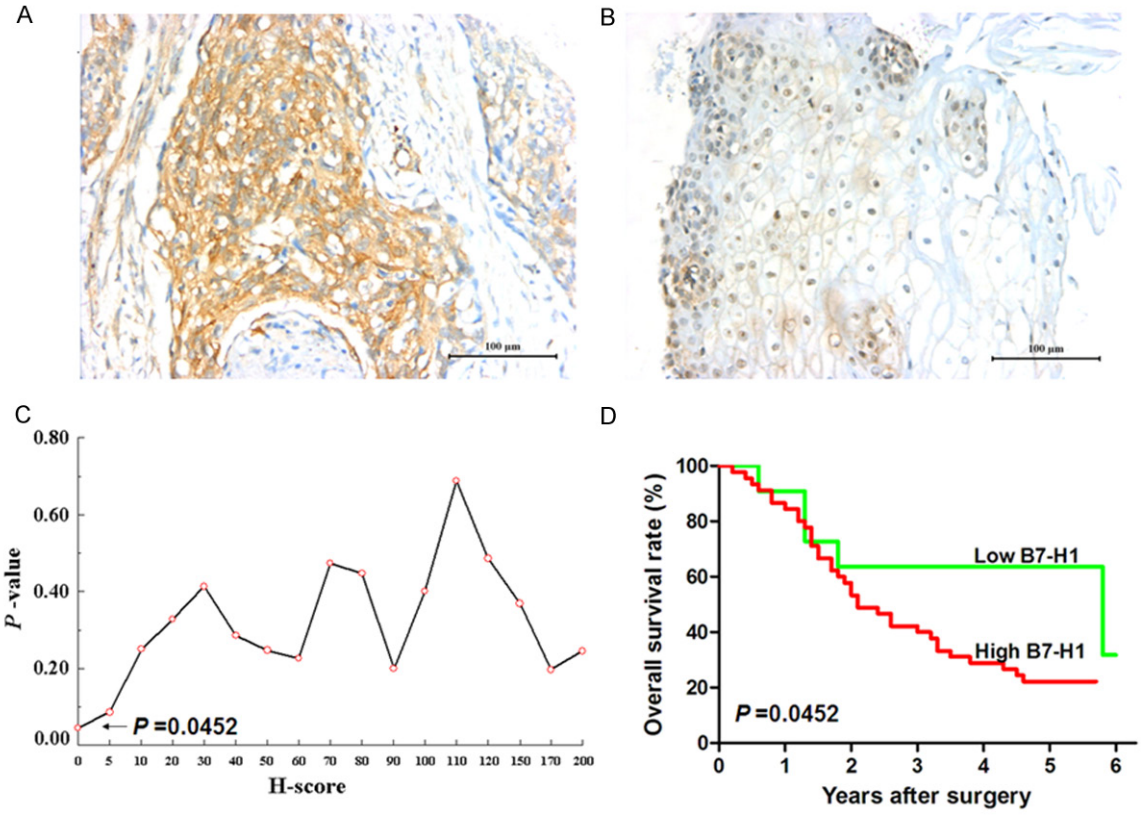

Figure 2. Survival analysis of membrane/cytoplasm B7-H1 expression in human esophageal cancer tissues. A. Positive membrane/cytoplasm B7-H1 expression on esophageal cancer tissue. B. Weakly expression of B7-H1 in epithelial cells of adjacent normal esophageal tissue. Scale bar=100 μm. C. The minimum *P*-value seek in the log-rank survival analysis of membrane/cytoplasm B7-H1 expression in human esophageal cancer tissues was performed, and when the cutoff value of *H-score* = 0 was selected, the minimal *P*-value = 0.0452 was found. D. The log-rank survival analysis was performed when *H-score* = 0, Hazard Ratio = 2.157, 95% CI: 1.017~4.577, *P* = 0.0452.

Table 1. It demonstrated that membrane and cytoplasm B7-H1 expression in human esophageal cancer tissues was significantly correlated with tumor invasion depth (*P* = 0.0261), where-

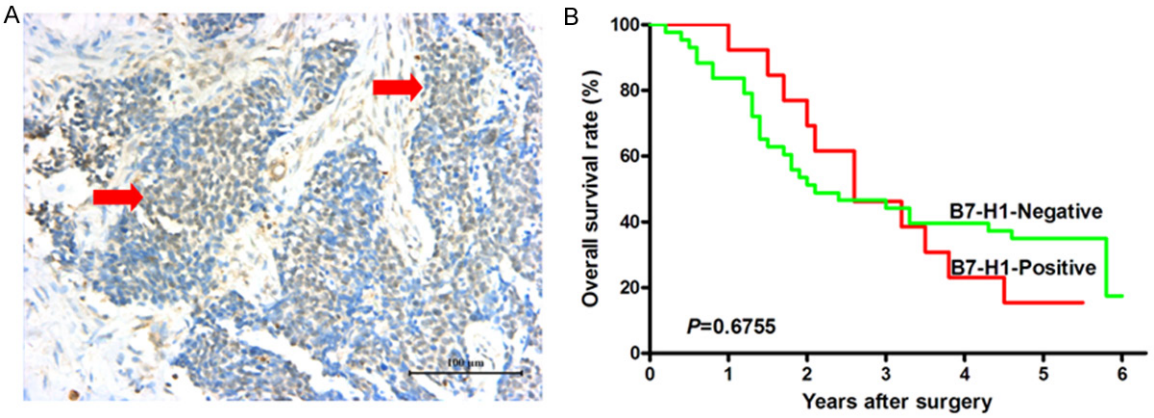

Figure 3. Survival analysis of B7-H1 expression in nuclei of cancer cells in human esophageal cancer tissues. A. Positive B7-H1 expression in nuclei of cancer cells in esophageal cancer tissue, scale bar = 100 μm. B. The log-rank survival analysis showed that there was no statistically significant difference in prognosis between the patients with positive nuclear B7-H1 staining and the patients with negative nuclear B7-H1 staining, $P = 0.6755$.

Table 2. Correlation between clinical parameters and B7-H1 expression in nuclei of cancer cells

Clinical parameters	Cases	B7-H1 staining			*P*-value
		Positive	Negative	χ^2	
Gender					
Male	76	61	15	0.0439	0.8340
Female	23	18	5		
Age (years)					
< 60	51	43	8	1.3310	0.2487
≥ 60	48	36	12		
Tumor size (cm)					
< 3.5	35	29	6	0.3143	0.5751
≥ 3.5	64	50	14		
Tumor invasion depth (T)					
$T_1 + T_2$	35	32	3	4.5430	**0.0331**
$T_3 + T_4$	64	47	17		
Nodal metastasis (N)					
Yes	34	29	5	0.9704	0.3246
No	65	50	15		
Distant metastasis (M)					
Yes	6	5	1	0.0495	0.8239
No	93	74	19		
TNM stage					
I	5	5	0	0.0143	0.9049
II	57	44	13		
III	31	25	6		
IV	6	5	1		

Values in bold signify $P < 0.05$.

as it was not correlated with patient's gender, age, tumor size, nodal metastasis, distant metastasis and TNM stage. When the *H-score* cut-off value = 0 was selected (**Figure 2C**), the survival analysis showed that the overall survival of the patients with positive B7-H1 membrane/cytoplasm expression was significantly poorer than that of the patients with negative B7-H1 membrane/cytoplasm expression (Hazard ratio = 2.157, 95% CI: 1.017-4.577, $P = 0.0452$, **Figure 2D**). Moreover, as shown in **Table 2**, we also analyzed the correlation between patients' clinical parameters and B7-H1 expression in nuclei of esophageal cancer cells, and it also demonstrated that nuclear B7-H1 expression in human esophageal cancer tissues was significantly correlated with tumor invasion depth ($P = 0.0331$), whereas it was not correlated with patient's gender, age, tumor size, nodal metastasis, distant metastasis and TNM stage. The log-rank survival analysis showed that there was no statistically significant difference in prognosis between the patients with positive nuclear B7-H1 staining and the patients with negative nuclear B7-H1 staining (**Figure 3B**).

Discussion

B7-H1 expression has been found in various human cancers, and its expression level significantly associated with patients' prognoses and a series of clinico-pathological parameters of the patients, as well as densities of infiltrating immune cell subsets, such as lung cancer [17, 18], gastric cancer [12, 13], pancreatic carcinoma [19, 20], breast cancer [21-23], ovarian cancer [24, 25], bladder cancer [26, 27], renal cell carcinoma [28], liver cancer [29], cervical

cancer [30], and *etc*. Although Ohigashi and his colleagues reported that B7-H1 expression could be found in esophageal cancer tissues by using immunohistochemistry in frozen section of human esophageal cancer tissues, and revealed that higher expression of B7-H1 predicted poorer survival of esophageal cancer patients [14]. In the present study, we further studied B7-H1 expression in human esophageal cancer cell lines, and also used tissue microarray to investigate the B7-H1 expression pattern in human esophageal cancer tissues and to analyze its clinical significance. Our results confirmed that membranous B7-H1 expression could be found in TE-1 cell line, and also could be found in esophageal cancer tissues by using tissue microarray and immunohistochemistry. We also showed the B7-H1 expression level in human esophageal cancer tissue was significantly associated with patient's prognosis and tumor invasion depth.

We have previously reported that higher expression of B7-H1 in human gastric cancer significantly associated with tumor size, invasion, lymph node metastasis and survival time of the patients [12]. In addition, we also reported that higher expression of B7-H1 in human colorectal cancer tissues significantly associated with densities of infiltrating T cells subsets, and also associated with the expansion of regulatory T cells [31]. However, recent accumulated evidence also indicated that the molecules from B7 family, such B7-H1 and B7-H3, could not only highly express on tumor cells, have a critical role in regulation of T-cell mediated antitumor response, but also have effect in the biological characteristics of the tumor cells themselves [32, 33]. Ghebeh *et al.* [34] reported that B7-H1 expression in breast cancer is strongly associated with high proliferative Ki-67-expressing tumor cells, suggesting that B7-H1 expression on cancer cells roles importantly in cancer cell proliferation and cancer progression. Shi *et al.* [32] also confirmed that B7-H1 expression in human colorectal cancer is significantly associated with patient's prognosis and is involved in the regulation of the proliferation and the invasion of colorectal cancer cells. Interestingly, B7-H1 could also serve as an anti-apoptotic receptor on cancer cells, which is a novel mechanism indicating that cancer cells can use a receptor on immune cells as a ligand to induce resistance to therapy [35].

Moreover, Cao *et al.* [33, 36] recently found that skin-specific over-expression of B7-H1 could induce carcinogenesis of squamous cell carcinoma, and in the B7-H1 transgenic mice, B7-H1 transgenic-derived keratinocytes and squamous cell carcinoma exhibited a significant reduction of E-cadherin, and an elevated expression of the transcription factors Slug and Twist, suggesting that B7-H1 over-expression in keratinocytes promotes the epithelial-mesenchymal transition and accelerates carcinogenesis. As we know, the epithelial-mesenchymal transition is a key step toward cancer metastasis, and is also an identified phenotypes of tumor invasion and metastasis in human esophageal cancer [37, 38]. In addition, the cells underwent epithelial-mesenchymal transition also showed some phenotypes of stem cells [39]. Mani *et al.* [39] reported that the induction of an epithelial-mesenchymal transition in immortalized human mammary epithelial cells contributed to the acquisition of mesenchymal traits and in the expression of stem-cell markers. Thus, in future, it's of great importance for us to study the correlation between up-regulation of B7-H1 expression and reduced E-cadherin and/or enhanced *Slug* and *Twist* transcription factors in human esophageal squamous cell carcinoma, and to elucidate detailed mechanism of B7-H1 over-expression in the induction of epithelial-mesenchymal transition of cancer cells, cancer invasion and metastasis.

The cancer cell expressed B7-H1 also linked between chemotherapy and cancer immunoresistance. Zhang *et al.* [40] showed that some chemopreventive agents could up-regulated surface B7-H1 expression on human breast cancer cell lines, and then promoted B7-H1-mediated T cell apoptosis. Previous studies demonstrated that B7-H1, expressed on tumor cells, can interact with PD-1 to reverse a signal, which results tumor cells resistance to apoptosis induction by both immune effectors and pro-apoptotic drugs [35]. Strikingly, *Ghebeh* et al. [23] also reported that doxorubicin could down-regulate the surface B7-H1 expression and up-regulate the nuclear B7-H1 expression, suggesting an important role of B7-H1 in anti-apoptosis of cancer cells by the means of nuclear transportation of this co-stimulatory molecule. In our present study, we also found B7-H1 staining in nuclei of esophageal cancer cells, and the ratio of patients with B7-H1 stain-

ing in nuclei in the group with advanced T stage was significantly higher than that in the group with early T stage, which showed that the nuclear transportation of B7-H1 expression promote the invasion of esophageal cancer cells, but the detailed mechanism merits further investigations in future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Changping Wu or Dr. Jingting Jiang, Departments of Tumor Biological Treatment and Oncology, The Third Affiliated Hospital of Soochow University, Changzhou 213003, Jiangsu, China. E-mail: wcpjjt@163.com (CPW); jiangjingting@suda.edu.cn (JTJ)

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