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*Original Article*

# Clinicopathological significance of checkpoint B7H4 expression in esophageal squamous cell carcinoma

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**Abstract:** Objective: Immune checkpoint inhibitors that enhance the antitumor T cells' responses have revolutionized the field of cancer immunotherapy. Checkpoint B7H4 play negative modulation in cellular immune response especially including tumor immunity. We focus on the expression of B7H4 in the esophageal squamous cell carcinoma (ESCC) tissues, to explore the relevance between the expression and its prognostic significance. Methods: Expression of B7H4 mRNA and B7H4 protein in the ESCC tissues and nearby normal tissues were detected by qRT-PCR and immunohistochemistry. Results: B7H4 mRNA in ESCC was significantly elevated compared with the adjacent normal esophageal mucosa (6.455±0.893 vs. 5.427±1.129, *P*=0.017); Positive B7H4 immunostaining was observed in 96 of 101 (95.1%) of ESCC tissue, notablely up-regulated compared with the adjacent normal tissues. Moreover, we divided all patients into two groups: a lower B7H4 expression group (41 patients) and a higher B7H4 expression group (60 patients). We found B7H4 expression in ESCC was highly correlated with patient's gender (*P*=0.048), lymph node metastasis (*P*=0.032), and AJCC clinical stage (*P*=0.029). Conclusion: B7H4 is overexpressed in ESCC tissues, suggesting it could be a potential indicator for prognosis of ESCC, and a new biomarker to predict malignant tumors progression and prognosis.

**Keywords:** Immune checkpoint, B7H4, ESCC, immunohistochemistry (IHC), qRT-PCR

## Introduction

Esophageal cancer (EC) is the 8th most common cancer with an estimated 456,000 new cases per year worldwide, and was the 6th leading causes of cancer-related mortality in the world [1, 2]. Esophageal squamous cell carcinoma is predominant histologic subtype and the major type of EC in China. As one of the highest incidence areas of ESCC, especially in Southeasten Shanxi Province of China, although there have been great efforts to develop methods for a better prognosis, the 5-year survival rate of EC patients is remains far from optimism [3].

Immune checkpoints are co-stimulate molecules in the immune system that either turn on or off a signal. Many cancers protect themselves from the immune surveillance by inhibiting the T cell signal. Since around 2010 [4, 5] inhibitory checkpoint molecules have been increasingly considered as new targets for cancer immunotherapy. Immune checkpoint inhibitors that enhance the antitumor T cells' responses have revolutionized the field of cancer immunotherapy. It is particularly urgent to search for checkpoint closely related to the invasion and metastasis of ESCC. Immune checkpoint blockade has emerged as one of the most promising strategies for tackling solid tumors [6]. B7H4 (B7x, B7S1 or VTCNI) is identified B7-family member by 3 separate laboratories [7-9]. The potential role of B7 family members in the diagnosis and treatment of tumor is becoming more and more obvious [10]. As an inhibitory checkpoint molecule, the expression of B7H4 in tumor tissues is considered to be one of the important mechanisms of immune tolerance of tumor, but its potential contributions to tumoral immune escape and therapeutic targeting have been less studied. Moreover, mRNA encoding B7H4 is widely expressed by peripheral tissues and (in an inducible manner)

hematopoietic cells [11], indicating that the abnormal expression of B7H4 in tumor cells presumably is one of the mechanisms of tumor immune escape. However, the clinicopathological significance of B7H4 expression in ESCC is inadequate. In this study, we investigated the expression of B7H4 mRNA and protein in ESCC by qRT-PCR and immunohistochemistry, and analyze the relationship between B7H4 expression and patients' clinicopathological parameters, survival rate and prognostic information in order to explore the immune escape mechanism of ESCC.

## Materials and methods

### *Tissues and patients history*

All the patients in our study were chosen for the first time diagnosis and underwent esophagectomy without radiotherapy, chemotherapy and immunotherapy. The tissues were obtained from 101 patients (73 female, 28 male; median age 59 years; range 36-81 years) who had undergone radical esophagectomy in the Department of Surgery in Heping Hospital affiliated Changzhi Medical College (Changzhi, Shanxi, China) from January 2006 to October 2008. The tissue samples were collected rapidly during the operation, which composed of liquid nitrogen snap-frozen specimens and paraffin blocks. 80 samples of the adjacent nonneoplastic mucosa were used as control, which were removed during the same resection, measuring 3-10 cm away from the edge of the main tumor lesion. The samples for qRT-PCR were collected from fresh ESCC and adjacent normal tissue group, 22 cases per group, and were stored in liquid nitrogen immediately as they were isolated.

All tissues, with a histopathologic diagnosis of ESCC and the adjacent nonneoplastic mucosa, were confirmed by two independent pathologists who were blinded to the original diagnosis. To ascertain no carcinoma, metaplasia, dysplasia, and atypical hyperplasia in the nonneoplastic mucosa tissue, we use the strict evaluation criteria to diagnose, and the chronic inflammation was allowed. In addition, clinicopathological parameters were collected including gender, age, tumor size, depth of invasion, cell differentiation, lymphovascular invasion and AJCC clinical stage. Tumors were staged according to the seventh edition of the AJCC staging system for ESCC [12].

The regular assessment of survival status was continuous after surgery until September, 2014. The mean follow-up period was 80±12 months (range, 3-99 months). We considered as uncensored only the records of patients who had died of ESCC, we considered as censored record of all patients who were alive at the end of follow-up interval or patients who died of a cause not related to ESCC. Follow-up records were made every month during the first year after surgery, then trimonthly during the second year, and once half a year thereafter. The study was approved by the Medical Ethics and Human Clinical Trial Committee of Changzhi Medical College.

### *RNA extraction and qRT-PCR*

23 frozen tissue tumor samples were chosen by using microscopy to ensure 90% of each sample composed of tumor and matched nonneoplastic esophageal mucosa specimens at least, which were also obtained at the same time (included in the former 101 pairs of ESCC and their corresponding nonneoplastic epithelia tissues). Total RNA of every tissue was prepared by using Primescript RT kit with gDNA eraser (Takara, Dalian China) is a special reverse transcriptase reagent that can remove genomic DNA for real time RT-PCR reaction, and RNA quality was verified by spectrophotometry prior to use. Two μg RNA was reverse-transcribed at 37°C for 15 min, 85°C for 5 sec, 4°C for 10 min in a 20 μl reaction mixture using the EnergicScript First Strand cDNA Synthesis Kits (ShineGene, Shanghai China). Genomic copy number of B7H4 was evaluated by qRT-PCR on a qTOWER2.2 Real-time PCR System (Analytik Jena, Germany) with SYBR Premix Ex TaqII Real Time qPCR Kit (Takara, Dalian China), according to the manufacturer's protocol. β-actin was applied as the internal reference. The primer sequences were designed with Primer premier 5.0 software based on the GeneBank accession numbers NM_001253849.1 (gene sequence of VTCN1, GeneID: 79679) and NM_001101.3 (gene sequence of β-actin, GeneID: 5245). The primers used for genomic VTCN1 detection were: 5'-TCTGGGCATCCCAAGTTGAC-3' (forward), 5'-TCCGCCTTTTGATCTCCGATT-3' (reverse). β-actin: 5'-TGACGGGACATCCGCAAAG-3' (forward), 5'-CTGGAAGGTGGACAGCAGG-3' (reverse). The size of the product was 193 bp and 205 bp respectively. Experiments were performed in triplicate in the same reac-

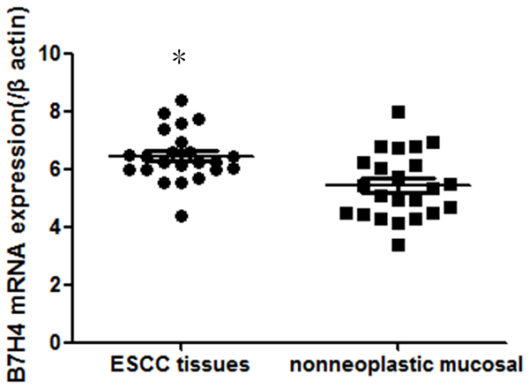


**Figure 1.** B7H4/β-actin mRNA expression level in ESCC tissues was significantly higher than that in the corresponding nonneoplastic esophageal mucosa tissues (*P*=0.017). **P*<0.05.

tion. Results were analyzed and relative fold-changes between transcript levels per the manufacturer's instructions. Results of the real-time PCR data are presented as C(t), which is defined as the threshold PCR cycle number at which an amplified product is first detected. The average C(t) was calculated for both VTCN1 and β-actin, and ΔC(t) was determined as the mean of the triplicate C(t)s for VTCN1 minus the mean of the triplicate C(t)s for β-actin. The relative copy number of VTCN1 for a sample compared with β-actin was expressed as 2-ΔC(t). Using this method, the data are presented as the folds change in the target sample of VTCN1 relative to β-actin [13].

Using Ct (Count of Threshold) the relative quantitative method value calculation and analysis of differentially expressed genes in different samples, the Ct value is in each reaction tube fluorescent signal (amplified) reach the amplification cycles through the domain value. Each sample corresponds to 3 holes, the average values of 3 holes is the Ct value; ΔCt is the target gene (VTCN1) average Ct value minus the average value of the corresponding reference gene template (β-actin) Ct value, the experimental group and the control group were repeated three times experiment; ΔΔCt value is the value of the experimental group ΔCt minus the control group ΔCt value $2^{-\Delta\Delta Ct}$ is the target gene expression in the experimental group compared with control group. The changes of times through the quantitative method, the relative expression can be compared to the experimental group and control group. Standard curves were established for each primer set and both reference and target reactions were performed for each sample.

*Immunohistochemistry*

The immunohistochemical reactions were performed on 4 μm thick paraffin tissue sections. For immunohistochemical analysis, all the slides were repaired in citrate buffer (pH 6.0) for 6 min; immersed in 3% hydrogen peroxide solution 37°C for 10 min; PBS washing three times, 3 min × 5 times; Then we blocked the slides with 10% normal goat serum at 37°C for 30 min and then incubated with B7H4 rabbit polyclonal antibody (1:300 dilution, Cell Signaling Technology, Massachusetts, USA) overnight at 4°C. After washed with phosphate buffer solution (PBS), the sections were treated with corresponding streptavidin-peroxidase conjugated second antibody (Zhongshan Golden Bridge Corporation, Beijing, China) then color reaction was detected by diaminobenzidine (DAB) reagent. Negative and positive controls were included for experiments. The gastric carcinoma specimens were utilized as positive control for B7H4. Both stains were performed with the same procedures; only the primary antibodies were replaced by PBS as negative controls.

*Slide evaluation of immunohistochemical staining*

Immunostaining for B7H4 was graded by a semiquantitative method based on a scale; both the distribution and intensity of the staining were considered. Evaluation of the IHC was performed by two pathologists, who were blinded to the original histological diagnosis. The result of the tissues was determined from at least 1,000 cells that were counted systematically at × 400 magnification in five visual fields. In the IHC test for B7H4, the presence of diffuse cytoplasm staining was considered to be significant. The intensity was scored as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positive cells was scored as follows: 1 (0-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The two scores were combined to obtain the final one: negative (0-3), weakly positive (4-5), or strongly positive (6-7) [13].

*Statistical analysis*

All analysis was performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA). The Chi-

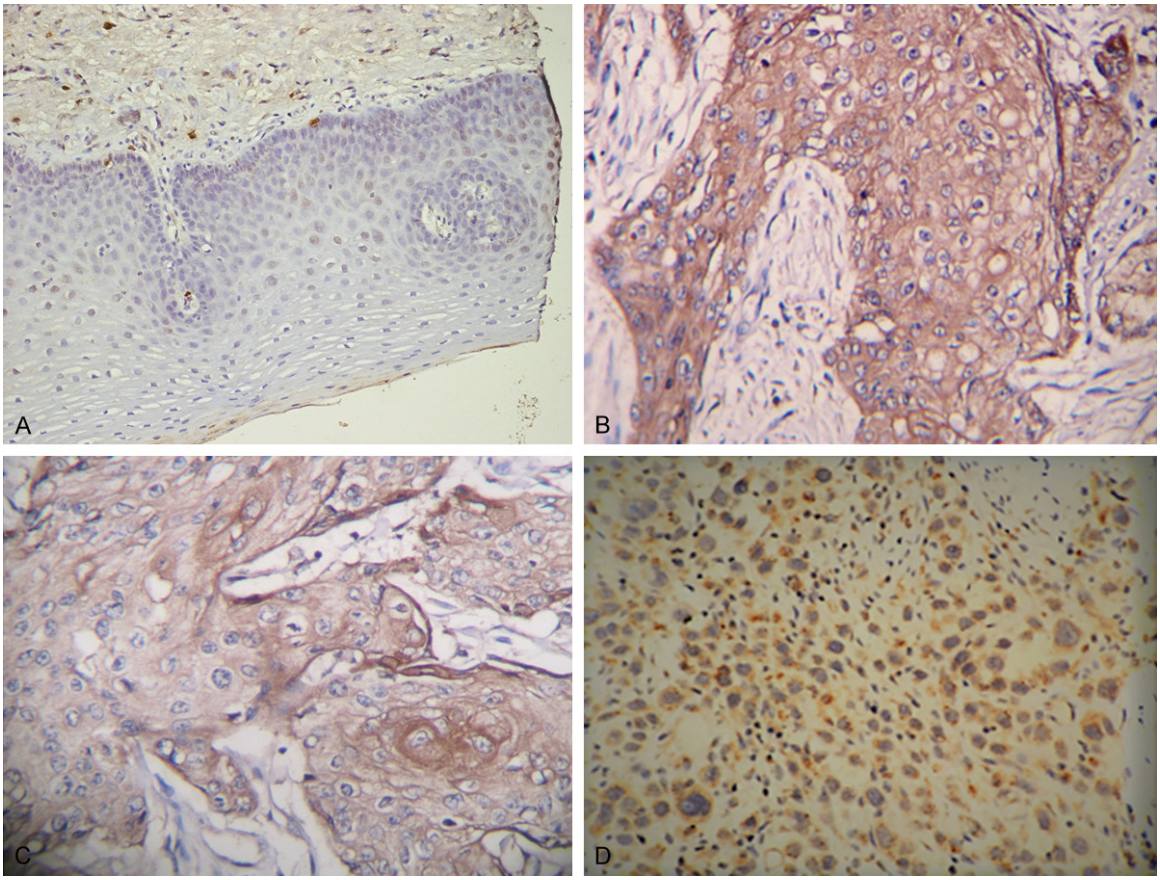


**Figure 2.** A. B7H4 expression in non-neoplastic esophageal tissue (SP × 200). B. B7H4 expression in ESCC (SP × 200). C. B7H4 expression in high grade differentiation of ESCC (SP × 200). D. B7H4 expression in low grade differentiation of ESCC (SP × 200).

square test was used to analyze the correlation between B7H4 expression and clinicopathological parameters. The data are expressed as mean ± standard deviation (SD). Kaplan-Meier method was used to draw survival curves; Comparative analysis of subgroups was determined with the log-rank test. Univariate proportional hazards regression was used to estimate the dependence of survival on each variable. Multivariate survival analysis was based on Cox proportional hazard model to test the variables selected by univariate analysis as having prognostic value. All statistical significance was considered for $P<0.05$.

## Results

*Experssion of B7H4 mRNA*

QRT-PCR was performed on 20 pairs of tumors and their nonneoplastic counterparts. The boxplots of QRT-PCR were shown in **Figure 1**. Compared to the nonneoplastic esophageal mucosal samples, B7H4 expression in the ESCC specimens were highly variable. The analysis revealed that the levels of B7H4/β-actin mRNA expression in ESCC tissues were significantly higher than those in the corresponding nonneoplastic esophageal mucosal tissues (6.455±0.893 vs. 5.427±1.129, $P=0.017$).

*Expression of B7H4 protein in ESCC and the relationship between B7H4 expression and patients' clinicopathological parameters*

As performed by IHC, B7H4 expression was found in cell cytoplasm, few in nuclei as well. The B7H4 expression in ESCC was significantly higher than in nonneoplastic esophageal tissues (**Figure 2A**, **2B**).

B7H4 expression was noted in 96 out of 101 (95.1%) cases of ESCC and 53 out of 80 (66.3%) cases of nonneoplastic esophageal

**Table 1.** Correlation of B7H4 expression with clinicapathological parameters

| Variable | | n | B7H4 expression (%) | | *P*-value |
|---|---|---|---|---|---|
| | | | High | Low | |
| Overall frequency | Nonneoplastic | 80 | 53 (66.3) | 27 (33.8) | 0.000* |
| | ESCC | 101 | 96 (95.1) | 5 (4.9) | |
| Gender | Male | 73 | 39 (53.4) | 34 (46.6) | 0.048* |
| | Female | 28 | 21 (75.0) | 7 (25.0) | |
| Age (yr) at surgery | ≥60 | 68 | 39 (57.4) | 29 (42.6) | 0.691 |
| | <60 | 32 | 17 (53.1) | 15 (46.9) | |
| Tumor size (cm) | <4 | 24 | 12 (50.0) | 12 (50.0) | 0.790 |
| | 4-7 | 71 | 41 (57.7) | 30 (42.3) | |
| | ≥8 | 5 | 3 (60.0) | 2 (40.0) | |
| Cell differentiation | High-grade | 27 | 16 (59.3) | 11 (40.7) | 0.120 |
| | Middle-grade | 39 | 21 (53.8) | 18 (46.2) | |
| | Low-grade | 35 | 19 (54.3) | 16 (45.7) | |
| Depth of invasion | T1 | 5 | 3 (60.0) | 2 (40.0) | 0.978 |
| | T2 | 13 | 7 (53.8) | 6 (46.2) | |
| | T3 | 79 | 44 (55.6) | 35 (44.4) | |
| | T4 | 3 | 2 (66.7) | 1 (33.3) | |
| Lymphatic invasion | (-) | 56 | 28 (50.0) | 28 (50.0) | 0.032* |
| | (+) | 45 | 32 (71.1) | 13 (28.9) | |
| AJCC clinical stage | I+II | 50 | 34 (68.0) | 16 (32.0) | 0.029* |
| | III+IV | 51 | 44 (86.3) | 7 (13.7) | |

*$P<0.05$.

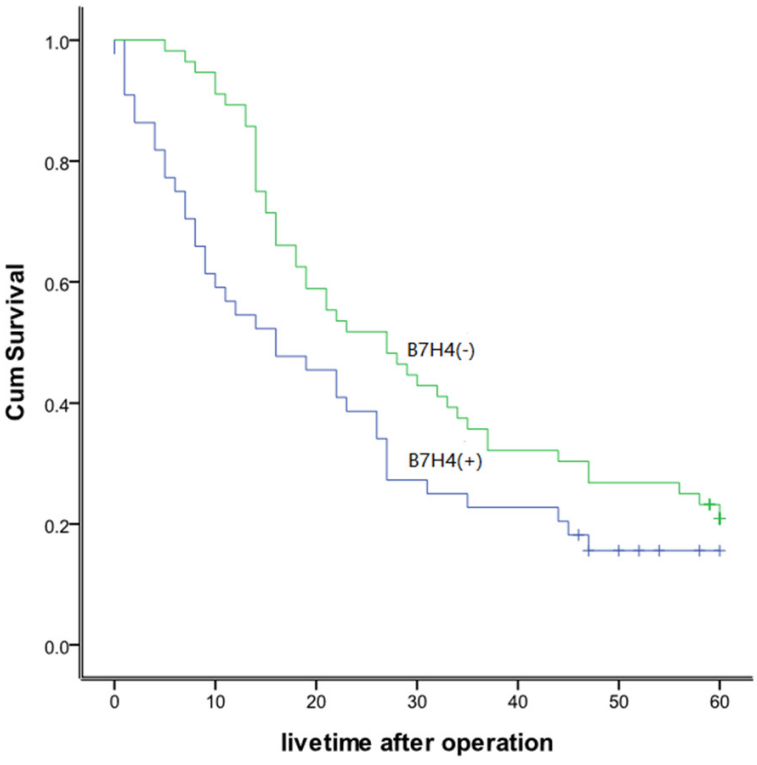


**Figure 3.** Overall survival curves of patients with ESCC according to the status of B7H4 expression ($\chi^2$=4.367, *P*=0.037).

tissues. A significant up-regulation of B7H4 immunoreactivity was found in ESCC compared to the nonneoplastic esophageal tissue ($\chi^2$= 25.010, *P*=0.000). The statistical analyses also show the relationship between B7-H4 expression and clinicopathological parameters. The percentage of B7H4 positive expression showed a significant difference in gender (*P*= 0.048), lymphatic invasion (*P*=0.032) and AJCC clinical stage (*P*=0.029). However, there were no statistically significant correlations between B7H4 expression and age (*P*=0.691), tumor size (*P*= 0.790), cell differentiation (*P*=0.120) (**Figure 2C**, **2D**), depth of invasion (*P*=0.978) (**Table 1**).

*Survival analysis*

In order to determine the prognostic value of B7H4, Kaplan-Meier survival curves for all 101 patients have demonstrated. The median survival time for patients with negative B7H4 expression was 23 (95% CI: 22.2-33.6) months compared to 16 (95% CI: 18.2-29.9) months for patients with positive B7H4 expression. The 1-year, 3-year and 5-year survival rate were 60.5%, 22.7% and 15.9% in the negative expression group (n=44) compared with 73.6%, 32.1% and 21.4% in the B7H4 positive group (n=57). The B7H4 positive expression group had significantly a worse survival rate than the negative group ($\chi^2$= 4.367, *P*=0.037) (**Figure 3**).

## Discussion

Immune checkpoint inhibitors that enhance antitumor T cell responses have revolutionized the field of cancer immu-

notherapy [14]. It is intriguing to groping how to boost the antitumor activity of immune cell populations. As tumor cell surface often lack of costimulatory molecules expression, the T lymphocytes deficiency of second signal activated molecules cannot be effectively activated, and the expression of some negative regulation of costimulatory molecule is high. However, cancer in human body may induce an immunosuppressive microenvironment that limits immune response to its antigens. To induce a potent antitumor immune response, one of the most promising immunotherapies is blockade of immune checkpoints, i.e. a group of costimulatory molecules negatively regulating the immune system. Their blockade would overcome immune tolerance in the tumor microenvironment and amplify antitumor immunity. What's more, immune checkpoint blockade may turn out even more profitable, as some of immune checkpoints and their ligands are expressed on tumor surface and on tumor infiltrating lymphocytes, contributing to the immunosuppressive cancer microenvironment. B7-H4 is able to negatively regulate T lymphocyte immune response as a kind of inhibitory checkpoint molecules [15]. It has been found that increased B7H4 expression is involved in shaping the tumor microenvironment, and aberrant B7H4 expression is associated with various clinicopathological features in many human malignancies. Recently studies have revealed that the expression of B7H4 mRNA in a variety of organizations. Jiang J et al. [16] detected the expression of B7H4 mRNA in gastric carcinoma was higher than adjacent normal tissue, which is consistent with our present results shows that B7H4 mRNA was significantly elevated compared with the adjacent normal esophageal mucosas (P=0.001) by qRT-PCR. These figures indicate B7H4 owns a potential to facilitate the therioma formation and associate with tumor formation, cell transformation and growth, tumor immune escape. However, using immunohistochemical to detect all normal tissues, the expression of B7H4 protein was not found. But the positive expression of B7H4 protein is 43% in non-small cell lung cancer [17], 59.1% in the renal cell carcinoma [18], 85% in ovarian cancer [19], 95.5% in ESCC [20]. In addition, the endometrial carcinoma 11, prostate cancer 12, gastric cancer 13, high expression of B7H4 in breast cancer [21] showed 14 other tumor tissues to study. Jiang J et al. [16] demonstrated that higher B7H4 expression in cancer cells was significantly associated with poor prognosis of the patients suffering from gastric cancer. The results show that the organism under normal conditions can at the level of translation on the expression of B7H4 in the strict regulation, but this occurred in the process of tumor control mechanism of the disorder. Their next study demonstrated that B7H4 expression in human ESCC is associated with cancer progression, reduced tumor immunosurveillance and worse patient outcomes [12]. B7H4 can serve as a novel prognostic predictor for human ESCC and a potential target for the immune therapy against this malignancy. Our study found that the expression of B7H4 was correlated with the sex, lymphatic invasion and AJCC clinical stage of ESCC, but not with differentiation degree. These findings suggest that B7H4 functions as an essential malignancy gene and facilitate immune escape of cancer as well. As a result, the expression of B7H4 might play a critical role in the tumorigenesis and tumor progression of ESCC. Analysis of survival data revealed that positive expression of B7H4 was associated with a poorer prognosis of ESCC, in the application of Kaplan-Meier survival curves, suggested that B7H4 protein could be a useful indicator for prognosis of ESCC.

In conclusion, our preliminary study demonstrates that B7H4 could be a helpful indicator that provides clinically useful prognostic information in cases of ESCC. Current evidence indicates that B7H4 could be an attractive biomarker for the occurrence and development of cancer. Therefore, further investigation is still required to explore the molecular mechanisms of B7H4 in ESCC. Eliminating, blocking or diminishing the B7H4 pathway may reestablish T-cell mediated antitumor effect and increase the survival of cancer patients. Therefore, targeting B7H4 has promising potential for improving the efficacy of immunotherapy for cancer. Ultimately the outcome of immunotherapy targeted at B7H4 should be assessed and evaluated in cancer patients in clinical trials. The mechanism of tumor escape being revealed, but due to the tumor multiplicity and complexity, there are still many problems to be studied further. How to find more targets, the activated immune cells, reversal the immune inhibitory state of the microenvironment, which is still a long jour-

ney for the future research in the field of tumor immunity.

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## Disclosure of conflict of interest

None.

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