The prognostic value of B7-H6 in esophageal squamous cell carcinoma

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Research article

Keywords: B7-H6, Esophageal squamous cell carcinoma, Immunohistochemistry, prognosis.

Posted Date: August 14th, 2019

DOI: <https://doi.org/10.21203/rs.2.12817/v1>

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Version of Record: A version of this preprint was published at Scientific Reports on December 1st, 2019.
See the published version at <https://doi.org/10.1038/s41598-019-54731-9>.

Abstract

Background B7-h6, a member of the B7 family molecules, participates in the clearance of tumor cells by binding to NKp30 on NK cells. The expression of B7-H6 in esophageal squamous cell carcinoma and the clinical significance is unknown. The goal of this study was to determine the expression of B7-H6 in esophageal squamous cell carcinoma and the clinical significance of B7-H6 expression. Patients and methods We retrospectively collected clinical data from 145 patients diagnosed with esophageal squamous cell carcinoma between January 2007 and December 2008. These patients had all previously undergone surgical treatment for esophageal cancer, were clearly diagnosed, and had not received chemotherapy or radiotherapy. In addition, pathological tissue samples from the 145 patients were collected to detect the expression of B7-H6 by immunohistochemistry. The chi-square (χ^2) test was used to analyse the relationships between B7-H6 and clinicopathological characteristics. The prognosis of the patients were analysed by Cox proportional hazards regression analysis and Kaplan-Meier analysis. Results B7-H6 was present in 133/145 (91.72%) of the esophageal squamous cell carcinoma samples and all localized in the cytoplasm. The expression level of B7-H6 was correlated with T stage ($P=0.036$) and lymphatic metastasis status ($P=0.044$). According to the results of the ROC curve analysis, H-score =90 was selected as the cut-off value. The 145 patients were divided into two groups, the high B7-H6 expression (H-score>90) group and the low B7-H6 expression (H-score $\leq$ 90) group. Cox proportional hazards regression analysis indicated that tumour size ($P=0.021$), B7-H6 expression ($P=0.025$) and lymphatic metastasis status ($P=0.049$) were independent prognostic factors for esophageal squamous cell carcinoma. Kaplan-Meier analysis with the log-rank test demonstrated that the patients with high B7-H6 expression ($P = 0.003$), lymphatic metastasis ($P < 0.001$) or a tumour size ≥ 3.0 cm ($P = 0.001$) had significantly worse survival than those with low B7-H6 expression, no lymphatic metastasis or a tumour size < 3.0 cm respectively. Conclusion B7-H6 is widely expressed in esophageal squamous cell carcinoma and high expression of B7-H6 can be used as a predictor of poor prognosis.

Background

Esophageal cancer is one of the most common malignant tumours in the world, ranking as the 6th most common cause of cancer-related death and the 8th most common cancer in the world, with an increasing incidence.(1) Esophageal cancer is the third most common cancer in Chinese men, and one of the top five in women, (2) with esophageal squamous cell carcinoma (ESCC) accounting for approximately 90% of esophageal cancer cases. Most patients are already in a relatively advanced stage with a poor prognosis when they are first diagnosed, and the overall 5-year survival rate is 15%~25%.(1) In recent years, although surgery, radiotherapy, chemotherapy, biological therapy and other comprehensive treatment methods have been widely used for the treatment of esophageal cancer, the prognosis and 5-year survival rate of esophageal cancer patients have remained very poor. Therefore, in-depth studies of the molecular mechanism underlying the occurrence and development of esophageal squamous cell carcinoma and the search for new molecular targets for diagnosis and prognostic monitoring have important clinical application value.

· Tumour immune escape is an important molecular mechanism in the processes of tumourigenesis, invasion and metastasis. Tumour cells are usually unable to provide effective antigen signals, or the body has a defective immune response and is immunosuppressed, resulting in tumour cells being able to resist attacks by immune effector cells and escape the body's immune monitoring. (3) In this process, costimulatory molecules and their regulatory networks play an important role. Costimulatory molecules are mainly divided into two superfamilies, B7/CD28 and TNF/TNFR. (4) Recently discovered new members of the B7 family of costimulatory molecules, such as B7-H1, B7-H3, B7-H4 and B7-H6, are widely expressed in many human tumour tissue types and can participate in the negative regulation of the T/natural killer (NK) cell-mediated antitumour immune response, thus attracting wide attention. (4, 5) Studies have revealed that the expression of PD-L1 (B7-H1) and B7-H3 in esophageal cancer is associated with patient prognosis. (6-8) Ling Wang et al reported B7-H3 and B7-H4 were widely expressed in esophageal squamous cell carcinoma tissues. High B7-H3 and B7-H4 expression was associated with advanced TNM stage and lymph node metastasis. Patients with both B7-H3 and B7-H4 high-expressed tumors had the poorest prognosis. (9) Lijie Chen et al demonstrated that [B7-H4 expression in esophageal squamous cell carcinoma is associated with cancer progression, reduced tumor immunosurveillance and worse patient outcomes](#). (10)

B7-H6 is a newly identified ligand in the B7 family. (11, 12) It is a type I transmembrane protein that shows considerable homology with the B7-H1 and B7-H3 proteins. (13) Studies have shown that B7-H6 can be activated by binding to the activating receptor NKp30 on the surface of NK cells and that B7-H6 promotes TNF- and IFN-mediated killing of target cells by NK cells, which is one of the important mechanisms of NK cell-mediated antitumour immunity. (13) B7-H6 mRNA expression was not found in 48 normal tissue samples under steady-state conditions, but several subsets of human primary lymphoma, leukaemia, ovarian cancer, brain tumours, breast cancers, renal cell carcinoma (RCC), and various sarcomas potentially express large amounts of B7-H6. (12) The expression level of the B7-H6 protein is significantly upregulated in cancer tissue compared with normal tissue, which is closely related to the clinicopathological characteristics and prognosis of patients. (14-17) However, until now, no reports have studied the clinical significance of B7-H6 expression in esophageal squamous cell carcinoma. In the present study, we used immunohistochemistry to investigate B7-H6 expression in esophageal squamous cell carcinoma tissue samples and to analyse the clinical implications of this expression.

Methods

Patient characteristics

We retrospectively collected clinical data from 145 patients with esophageal squamous cell carcinoma between January 2007 and December 2008. These patients had all undergone surgical treatment for esophageal cancer, were clearly diagnosed, and had not received chemotherapy or radiotherapy. In addition, pathological tissue samples from the 145 patients were collected to detect the expression of B7-H6 by immunohistochemistry. In addition, 7 non-malignant esophageal tissue samples from the non-malignant portion of the esophagus were collected and used as controls. The clinicopathological data of

all patients were available and were included in the statistical analysis. This study was approved by the Ethics Committee of the Sun Yat-sen University Cancer Center and written informed consent was provided by all patients based on the Declaration of Helsinki.

Antibodies and major reagents

A rabbit anti-human B7-H6 polyclonal antibody (ab121794) was purchased from Abcam (Cambridge, MA, USA; dilution 1/100), and a horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit secondary antibody was purchased from Dako (Glostrup, Denmark). A DAB colour developing kit was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China; cat. no. zli-9017).

Immunohistochemistry

Paraffin-embedded tissue samples were cut into 5 µm serial sections and baked for 30 minutes in a 60°C constant-temperature box. The sections were deparaffinized with xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution for 10 minutes, and antigen retrieval was performed at 100°C for 20 minutes in a sodium citrate buffer solution (0.01 mmol/L, pH 6.0). After being soaked in distilled water for 10 minutes, the sections were incubated with 10% foetal bovine serum to block nonspecific binding. Next, the sections were incubated with the rabbit anti-human B7-H6 polyclonal antibody at 4°C overnight and then incubated with the HRP-conjugated goat anti-mouse/rabbit secondary antibody at room temperature for 60 minutes. The excess secondary antibody was removed by washing with TBS and developed with DAB colourant; the sections were stained with haematoxylin, dehydrated with an alcohol gradient, dried and sealed with neutral resin.

Evaluation of immunohistochemical (IHC) staining

The esophageal squamous cell carcinoma tissue samples were examined by two independent senior pathologists who were not informed of the patients' clinicopathological characteristics. The B7-H6 immunohistochemical staining results were analyzed according to a previously described method: (10, 18) $H\text{-score} = (\% \text{ tumour cells unstained} \times 0) + (\% \text{ tumour cells stained weakly} \times 1) + (\% \text{ tumour cells stained moderately} \times 2) + (\% \text{ tumour cells stained strongly} \times 3)$. The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), or "3" (strongly stained). The H-scores ranged from 0 (100% negative tumour cells) to 300 (100% strongly stained tumour cells). The results from the two pathologists were averaged and used in the statistical analyses.

Statistical analyses

Overall survival (OS), which was defined as the time from surgery to patient death or last follow-up, was used as a measure of prognosis. The final follow-up date was December 24, 2018. SPSS software (version 13.0; IBM Corp., Armonk, NY, USA) was used for data analysis. The correlation between the B7-H6 expression level and different clinicopathological parameters was analysed by the chi-square (χ^2) test, and survival data were analysed by univariate and multivariate Cox regression analyses, receiver

operating characteristic (ROC) curve analysis, and Kaplan-Meier analysis with the log-rank test. Statistical significance was defined at $P < 0.05$.

Results

Study population

Patient characteristics are presented in Table 1. The median age of the study population was 60 years (range from 34 to 88 years). There were 82 cases (56.6%) of grade I-II disease and 63 cases (43.4%) of grade III-IV disease, according to TNM staging. Of the 145 patients examined, 79 (54.5%) died before the end of the observation period.

B7-H6 expression in esophageal tissue

To determine the expression levels of the B7-H6 protein in esophageal squamous cell carcinoma tissue, immunohistochemical analysis was performed (Figure 1). This approach revealed that B7-H6 was present in 133/145 (91.72%) of the esophageal squamous cell cancer tissue samples. B7-H6 was always localized in the cytoplasm of the esophageal tumour cells, while weak B7-H6 staining was found in non-malignant esophageal tissue. In the 145 samples of esophageal squamous cell carcinoma, the median, maximum and minimum values of the B7-H6 staining H-score were 40, 180 and 0, respectively.

Relationships between B7-H6 and clinicopathological characteristics

The associations between the clinicopathological characteristics of the patients with esophageal squamous cell carcinoma and B7-H6 expression are presented in Table 2. To investigate the associations between the clinicopathological characteristics of esophageal cancer and B7-H6 protein expression, the 145 patients were divided into two major subgroups according to the intensity of the B7-H6 immunohistochemical staining as follows: $0 \leq \text{H-score} < 40$ and $\text{H-score} \geq 40$ (40 was the median value). This stratification revealed that B7-H6 expression was significantly correlated with T stage ($P = 0.036$) and lymphatic metastasis status ($P = 0.044$); however, B7-H6 expression was not associated with other clinicopathological parameters, including gender, age, tumour size, tumour location, differentiation degree, TNM stage, local recurrence status and metastasis status ($P > 0.05$).

Association between B7-H6 expression and patient prognosis

With H-score = 90 as the cut-off value, the 145 patients with esophageal squamous cell carcinoma were divided into two groups: a low B7-H6 expression group ($\text{H-score} \leq 90$) and a high B7-H6 expression group ($\text{H-score} > 90$). Univariate and multivariate Cox regression analyses of the 145 patients showed that tumour size ($P = 0.021$), B7-H6 expression ($P = 0.025$) and lymphatic metastasis status ($P = 0.049$) were independent prognostic factors in esophageal squamous cell carcinoma, as shown in Table 3. To further study the prognostic value of tumour size, lymphatic metastasis status and B7-H6 expression in human esophageal squamous cell carcinoma, Kaplan-Meier analysis with the log-rank test was performed. The survival analysis demonstrated that the patients with high B7-H6 expression ($P = 0.003$, median survival

time of 29.9 months, Figure 2A), lymphatic metastasis ($P < 0.001$, median survival time of 23.8 months, Figure 2B) or a tumour size greater than or equal to 3.0 cm ($P = 0.001$, median survival time of 44.0 months, Figure 2C) had significantly worse survival than those with low B7-H6 expression (median survival time of 117.3 months), no lymphatic metastasis (median survival time was not achieved) or a tumour size less than 3.0 cm (median survival time was not achieved), respectively.

Discussion

The B7 family and the corresponding CD28 receptor family of costimulatory and coinhibitory molecules have previously been demonstrated to serve potential roles in the immune response, and these molecules have been revealed to be effective diagnostic markers and therapeutic targets in cancer. (4, 19) The newly discovered member of the B7 family B7-H6 interacts with its receptor on NK cells, namely, NKp30, and plays an important role in NK cell-mediated immune responses. (12) Abnormal B7-H6 expression is found in many human cancer types, suggesting that B7-H6 has important clinical significance.

The present study provides the first investigation into the relationship between the prognostic and clinical value of B7-H6 expression in esophageal squamous cell tissue. Immunohistochemical staining demonstrated that B7-H6 expression was present in most esophageal cancer tissue samples (91.72%, 133/145) and was always localized in the cytoplasm of tumour cells, which is consistent with the findings of other studies. (14, 17, 20) We also found that the expression level of B7-H6 correlated with T stage and lymphatic metastasis status but not with other clinicopathological parameters, which demonstrated that B7-H6 expression might be a marker to identify the T stage and lymphatic metastasis status of esophageal squamous cell carcinoma. This result is similar to the findings of studies in gastric carcinoma, ovarian cancer, non-small cell lung cancer, astrocytoma, breast cancer and other cancers. (14, 15, 17, 20-22) In addition, Cox regression analysis and the log-rank test revealed that tumour size, lymphatic metastasis status and B7-H6 expression were independent prognostic factors for esophageal squamous cell carcinoma. Patients with a tumour size greater than or equal to 3.0 cm, high B7-H6 expression or lymphatic metastasis had significantly worse survival than those with a tumour size less than 3.0 cm, low B7-H6 expression or no lymphatic metastasis, respectively, suggesting that a tumour size greater than or equal to 3.0 cm, high B7-H6 expression and lymphatic metastasis are predictors of a poor prognosis. This result is similar to data from other researchers. (15, 21, 22) Thus, B7-H6 may be regarded as a possible biomarker for predicting the OS time of esophageal squamous cell carcinoma patients, and also as an independent prognostic index for patients. In the past three years, some studies of the knockdown of B7-H6 expression in tumours have been carried out (16, 23, 24) and indicated that B7-H6 may be a potential therapeutic target in several human cancers. Therefore, it is believed that further studies on the expression of B7-H6 at the gene level and the knockdown of B7-H6 expression will also have certain clinical value in determining the prognosis of esophageal squamous cell carcinoma patients.

Conclusions

In recent years, immune checkpoint inhibition with antibodies that block cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have led to meaningful improvements in survival and have brought tumour immunotherapy into a new era. (25, 26) Several clinical studies in esophageal cancer using an anti-PD-1 monoclonal antibody, such as nivolumab or pembrolizumab, are in progress with recent promising results. (27-29) However, the relationship between PD-1/PD-L1 expression in esophageal cancer tissue and prognosis remains controversial. To date, no good biomarker has been found to guide treatment and prognosis. Our present study indicates that B7-H6 is widely expressed in esophageal squamous cell carcinoma tissue and that its expression level is significantly related to the prognosis of patients. The expression level of B7-H6 may be regarded as a prognostic marker in esophageal squamous cell carcinoma patients.

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Declarations

Acknowledgments

The authors would like to thank the patients who participated in this study, and two independent senior pathologists for technical support.

Funding

This study was supported by the Science and Technology Planning Project of the Guangdong Esophageal Cancer Research Institute (No Q201607) and 2016 youth teacher training program of sun yat-sen university (No 051703).

Disclosure

The authors have no potential conflicts of interest disclose.

Availability of data and materials

The dataset supporting the conclusions of this article is available in the repository [the Research Data Deposit public platform], [RDDA2019001075 in <http://www.researchdata.org.cn>].

Authors' contributions

S.Y and X.C conceived the original idea and designed the study. H.Z and K.W collected the data for the study, which were analysed by H.Z, J.D, and L.G. The data interpretation and manuscript drafting were performed by H.Z and J.D. The manuscript was revised by X.W, L.G, X.C, and S.Y. All authors reviewed the manuscript and gave the final approval for submission.

Tables

Table 1. Clinical characteristics of 145 patients

Characteristic	Number (%)
Age(Years)	
Median	60
Range	34-88
<60	71(49.0)
≥60	74(51.0)
Gender	
Male	107(73.8)
Female	38(26.2)
Tumor size (cm)	
≤ 3.0	69(47.6)
≥3.0	75(51.7)
Tumour location	
Upper third	14(9.7)
Middle third	90(62.1)
Lower third	41(28.3)
Differentiated degree	
Low	29(20.0)
Middle	60(41.4)
High	32(22.1)
Low-middle	13(9.0)
Middle-high	11(7.6)
T stage	
T1	13(9.0)
T2	23(15.9)
T3	96(66.2)
T4	13(9.0)
Lymphatic metastasis	
Yes	64(44.1)
No	81(55.9)
TNM stages	
I+II	82(56.6)
III+IV	63(43.4)
Death	
Yes	79(54.5)
No	66(45.5)

Table 2. Correlation between the B7-H6 expression level and the patients' clinical parameters

Clinical parameters	Cases	B7-H6 expression level		P-value
		H-score<40	H-score≥40	
Gender				0.096
Male	107	51	56	
Female	38	17	21	
Age(Years)				0.055
<60	71	34	37	
≥60	74	34	40	
Tumour size ,cm				0.652
< 3.0	69	35	34	
≥3.0	75	33	42	
Tumour location				0.66
Upper third	14	8	6	
Middle third	90	41	49	
Lower third	41	19	22	
Differentiated degree				1.389
Low	29	14	15	
Middle	60	30	30	
High	32	13	19	
Low-middle	13	5	8	
Middle-high	11	6	5	
T stage				8.526
T1	13	11	2	
T2	23	9	14	
T3	96	43	53	
T4	13	5	8	
Lymphatic metastasis				4.062
Yes	64	24	40	
No	81	44	37	
TNM stages				0.73
I+II	82	41	41	
III+IV	63	27	36	
Local recurrence or metastasis				2.794
Yes	30	10	20	
No	115	58	57	

Note: ^aP<0.05.

Table 3. Univariate and multivariate analyses of prognosis in esophageal squamous cell carcinoma

	Univariable		Multivariable	
	HR (95% CI)	P value	HR (95% CI)	P value
Age, years (<60 vs ≥60)	1.043 (0.670-1.621)	0.853		
Gender (male vs female)	0.814 (0.503-1.315)	0.400		
Tumour location				
Upper third vs Lower third	1.192(0.528-2.693)	0.673		
	0.891(0.535-1.484)			
Middle third vs Lower third		0.658		
Tumor size, cm (<3.0 vs ≥3.0)	2.095(1.323-3.316)	0.002 ^a	1.749(1.089-2.807)	0.021 ^a
Pathological stage				
T1 vs T4	0.161 (0.044-0.587)	0.006 ^a	0.400(0.092-1.745)	0.223
T2 vs T4	0.400 (0.169-0.948)	0.037 ^a	0.507(0.140-1.842)	0.302
T3 vs T4	0.567(0.288-1.118)	0.101	0.580(0.258-1.305)	0.188
Lymphatic metastasis (yes vs no)	2.914(1.856-4.577)	<0.001 ^a	2.157(1.002-4.644)	0.049 ^a
TNM stages (I-II vs III-IV)	2.912 (1.849-4.586)	<0.001 ^a	1.186 (0.474-2.969)	0.715
B7-H6 expression level (low vs high)	2.053(1.266-3.329)	0.004 ^a	1.751(1.071-2.861)	0.025 ^a

Note: ^aP<0.05.

Figures

Figure 1

Immunohistochemical staining (magnification: left, 5 $\times$; right, 100 $\times$). Notes: Immunohistochemical staining was used to detect B7-H6 expression (brown) in esophageal squamous cell carcinoma tissue samples and adjacent normal tissue samples. (A–B) Negative B7-H6 expression in esophageal squamous cell carcinoma tissue samples. (C–D) Low B7-H6 expression in esophageal squamous cell carcinoma tissue samples. (E–F) High B7-H6 expression in esophageal squamous cell carcinoma tissue samples. (G–H) Low B7-H6 expression in adjacent normal tissue samples.

Figure 2

Kaplan-Meier survival analysis of the associations between overall survival and tumour size (Figure 2A, $P=0.001$), lymphatic metastasis status (Figure 2B, $P<0.001$) or B7-H6 expression (Figure 2C, $P=0.003$) in patients with esophageal squamous cell carcinoma.