Int J Clin Exp Pathol 2016;9(9):9290-9296
www.ijcep.com /ISSN:1936-2625/IJCEP0032209

Original Article

B7-H4 expression and Treg cells in diffuse large B cell lymphoma: associations with patient outcome and clinical significance

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Received May 15, 2016; Accepted June 25, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Increasing evidence has confirmed the critical role of tumor immune microenvironment in the development of diffuse large B cell lymphoma (DLBCL). The aim of present study was to examine the expression of B7-H4 and Treg cells in DLBCL tissues, and evaluate the corresponding clinical significance. B7-H4 and Foxp3 expression in DLBCL tissues was examined by immunohistochemistry. Their relationship with clinicopathological factors and prognosis was statistically analyzed. Our findings demonstrated that B7-H4 and Foxp3 expression were meaningfully higher in tumor tissues than those in adjacent tissues (58.03% *vs.* 21.43%, *P*<0.001; 42.85% *vs.* 25.89%, *P*<0.001; respectively). B7-H4 levels were significantly correlated with tumor stage and distant metastasis (*P*=0.047, *P*=0.018, respectively), while Foxp3 levels were only significantly associated with extranodal sites (P=0.005). In addition, B7-H4 was positively correlated with Foxp3 in DLBCL tissues (r=0.22, P=0.019). Additionally, high B7-H4 levels or Foxp3 levels were significantly related to poor overall survival (*P*=0.019, *P*=0.001, respectively). Cox regression multivariate analysis confirmed that high B7-H4 or Foxp3 levels were independent prognostic factors (P=0.002 and P=0.008, respectively). Collectively, our data suggest that B7-H4 and Foxp3 may be useful as a prognostic factor and for diagnosis of DLBCL.

Keywords: B7-H4, Treg, Foxp3, Diffuse large B cell lymphoma

Introduction

Diffuse large B cell lymphoma (DLBCL), a heterogeneous category of aggressive lymphomas, is the most common type of non-Hodgkin lymphoma (NHL). Recently, evidence supporting a role for the tumor immune microenvironment in the development of DLBCL, suppressed immune function may influence the growth of lymphoma, and immune escape exerts an important effect on tumor progression [1, 2]. The immunoresponse was also identified as an significant prognostic factor for survival in DLBCL [3]. Recent research has confirmed the crucial role of B7 family members and their receptors in the regulation of tumor immune responses, including leukemias and lymphomas [4].

B7-H4 is a newly identified immunoregulatory member of the B7 family [4]. Studies have shown that B7-H4 signaling implicated in the T cell-mediated immune response through inhibiting T cell proliferation, cytokine secretion and the development of cytotoxicity [5]. Aberrant B7-H4 expression was found in a variety of malignant tumors, including lung cancer, liver cancer, breast cancer, ovarian cancer, pancreatic cancer and gastric cancer, but not in any of the normal tissues [6]. In addition, its expression was found to be closely associated with progression and prognosis of these malignancies [6].

Regulatory T (Treg) cells, which are characterized by expression of Foxp3, are a distinctive T cell subset with immunosuppressive capability. Treg cells play an important role in maintaining the stability of the immune system, and tumor immune tolerance, and may also reduce the immune response to lymphoma [7, 8]. Previous studies have shown that DLBCL had increased

infiltrating PD-1^{hi} Treg cell populations, and associated with poor prognosis in DLBCL [9]. However, the expression pattern and the prognostic value of B7-H4, as well as the correlation between B7-H4 expression and Treg cells in DLBCL has not yet been fully elucidated.

In the current study, we measured the B7-H4 and Foxp3 expression intensity in DLBCL surgical specimens using immunohistochemistry, and investigated the correlation between the expressions of these two proteins. Additionally, their correlation with clinicopathologic features, and the prognostic values of these two proteins in DLBCL was also evaluated.

Materials and methods

Patients, specimens and follow-up

This study was approved by the Research Ethics Committee of Wenzhou Central Hospital, P. R. China. Written informed consent was obtained from all of the patients according to the committee's regulations. 112 paraffin-embedded tumor tissues and paired adjacent tissues from DLBCL patients treated between 2008 and 2010 in the Department of Hematology, Wenzhou Central Hospital were obtained. All tissue sections were independently determined by two pathologists by histopathological examinations. There were 67 males and 45 females, with a median age of 61 (rang: 22-81) years, 52 patients were in stage I-II, 60 patients in stage III-IV. Clinicopathological characteristics of DLBCL patients were detailed in **Table 1**. All patients were followed up until Jun 2015, the median follow-up time was 56.6 months (range 4-70 months).

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens were cut into 4 μm-thick sections and mounted on glass slides. Immunohistochemistry streptavidin peroxidase (SP) conjugated method was used to detect the expression of B7-H4 and Foxp3 as described previously [10]. Briefly, antigen retrieval was performed by pressure cooking in PH 6 citrate buffer. After rinsing with PBS, Sections were incubated with the primary antibody overnight at 4°C. Mouse anti-human B-7-H4 monoclonal antibody (clone MIH43, 1:500 dilution), mouse anti-human FOXP3 monoclonal antibody (clone mAbcam 22510, 1:500 dilution) was purchased from Abcam (Cambridge, MA, USA). After washing with PBS, the sections were incubated with secondary biotinylated antibody at 37°C for 10 minutes. SP complex was added and the sections were visualized by incubating with DAB-H_2O_2 for 5-10 min, desired color reaction was observed when monitored with the microscope. All the slides were counterstained with hematoxylin. Nega-tive controls were performed by replacing the specific primary antibody with PBS.

The intensity of positive staining was measured using a computerized image system (Leica Microsystems Imaging Solutions, United Kingdom). Five fields were randomly selected, and three slides for each specimen were counted. The staining extent was scored from 0 to 3 based upon the percentage of positive cells (0, <5%; 1, five%-25%; 2, 25%-50%; 3, >50%). The intensity of staining was classified as follows: 0 point, no staining; 1 point, weak staining (light yellow); 2 points, moderate staining (brown); 3 points, strong staining (yellowish brown), respectively. The final score of B7-H4 expression was calculated using the percent of positive cell score × staining intensity, ranging 0-9. High B7-H4 or Foxp3 expression level was defined as a total score ≥4, and low B7-H4 or Foxp3 expression level as a total score <4.

Statistical analysis

Statistical analysis was performed with SPSS 19.0 for Windows (SPSS, Chicago, IL).

Data were expressed as means ± standard deviation (SD). The relationships between B7-H4, Foxp3 expression and clinicopathological characteristics were analyzed using χ^2 test. Correlation between B7-H4 and Foxp3 was performed using the Spearman's correlation coefficient. Survival curves were plotted using the Kaplan-Meier product-limit method, and differences between survival curves were using the log-rank test. Overall survival (OS) was defined as the time between the moment of diagnosis and death or the last follow-up. The Cox regression analysis in a forward stepwise method was used to evaluate the effect of multiple independent prognostic factors on survival outcome.

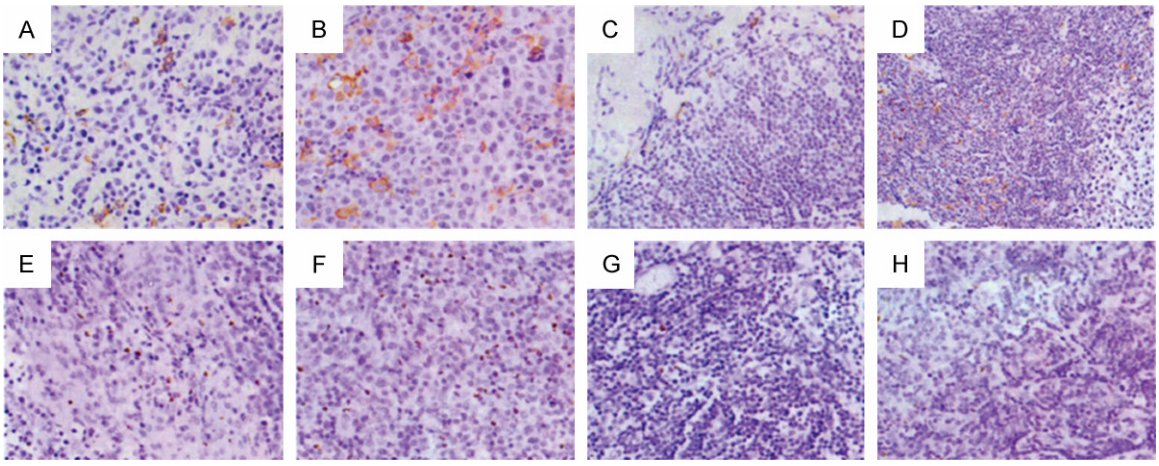

Figure 1. Immunohistochemical staining of B7-H4, Foxp3 in DLBCL tissues and adjacent tissues. A. Low B7-H4 expression in DLBCL tissues. B. High B7-H4 expression in DLBCL tissues. C. Low B7-H4 expression in adjacent tissues. D. High B7-H4 expression in adjacent tissues. E. Low Foxp3 expression in DLBCL tissues. F. High Foxp3 expression in DLBCL tissues. G. Low Foxp3 expression in adjacent tissues. H. High Foxp3 expression in adjacent tissues. (×400 magnification).

Differences were considered to be statistical significant when *P* value was less than 0.05.

Results

Expression of B7-H4 and Foxp3 in DLBCL

In the DLBCL tissues, B7-H4 was mainly expressed in the cytoplasm of the cancer cells, while Foxp3 expression was located within the T cell nucleus, and the cytoplasm and nucleus of cancer cells (**Figure 1**). Among the 112 DLBCL specimens, B7-H4 was high expressed in 58.03% (65/112) of DLBCL tissues, which was significantly higher than the 21.43% (24/112) in adjacent tissues ($P<0.001$); while Foxp3 was high expressed in 42.85% (48/112) of DLBCL tissues, which was significantly higher than the 25.89% (29/112) in adjacent tissues ($P<0.001$).

Correlation between B7-H4 and Foxp3 expression as well as clinicopathological features of DLBCL

The relationship between B7-H4, Foxp3 expression in DLBCL tissues and clinicopathologic features was summarized in **Table 1**. B7-H4 levels were significantly correlated with tumor stage ($P=0.047$) and extranodal sites ($P=0.018$). Foxp3 levels were significantly correlated with extranodal sites ($P=0.005$). The levels of B7-H4 and Foxp3 were not correlated with age, gender, pre-treatment LDH level and IPI score. In addition, spearman's rank correlation analysis showed that B7-H4 was positively correlated with Foxp3 in DLBCL tissues ($r=0.22$, $P=0.019$; **Figure 2**).

The prognostic significance of B7-H4 and Foxp3 in DLBCL

To the end of follow-up, 59 patients died. The 5-year overall survival rates were 47.7%; the median survival time was 29.8 months. Kaplan-Meier survival analysis showed that DLBCL patients with high B7-H4 levels showed lower OS rates than those with low B7-H4 levels (**Figure 3A**, log rank, $P=0.019$). The median survival time for high B7-H4 expression was 17.0 months while for low B7-H4 expression 33.7 months. In addition, DLBCL patients with high Foxp3 levels also showed lower OS rates than those with low B7-H4 levels (**Figure 3B**, log rank, $P=0.001$). The median survival time for high B7-H4 expression was 14.4 months while for low B7-H4 expression 35.4 months. Based on the B7-H4 and Foxp3 expression levels, 112 patients were divided into three groups: high B7-H4 and Foxp3 (Group I, n=41), high B7-H4 and low Foxp3, or low B7-H4 and high Foxp3 (Group II, n=30), and low B7-H4 and low Foxp3 (Group III, n=41). As showed in Figure 3C, the median survival time for group I was 13.4 months, significantly shorter than group II (26.5 months, $P<0.001$) and group III (50.1 months, $P<0.001$). Univariate and multivariate analyses

Table 1. Correlation between serum B7-H4, Foxp3 levels and clinicopathological features of 112 DLBCL patients

Characteristics	Cases (112)	B7-H4			Foxp3		
		Low	High	*P*-value	Low	High	*P*-value
Gender							
Male	67	29	38	0.730	39	28	0.781
Female	45	18	27		25	20	
Age (years)							
<60	55	22	33	0.679	31	24	0.870
≥60	57	25	32		33	24	
Stage							
I-II	52	27	25	0.047	33	19	0.208
III-IV	60	20	40		31	29	
Pre-treatment LDH							
Normal	52	21	31	0.752	27	25	0.299
Elevated	60	26	34		37	23	
Extranodal sites							
0-1	52	28	24	0.018	37	15	0.005
≥2	60	19	41		27	33	
IPI score							
0-2	76	31	45	0.714	44	32	0.909
2-4	36	16	20		21	16	

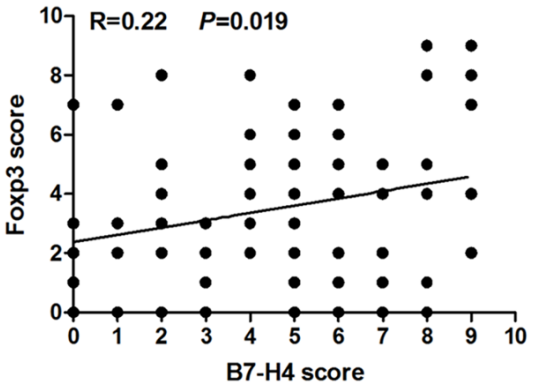

Figure 2. Correlation between B7-H4 and Foxp3 expression in DLBCL tissues. r, spearman's correlation coefficient.

showed that tumor stage, B7-H4 and Foxp3 were independent prognostic factors (*P*=0.013, *P*=0.002 and *P*=0.008, respectively; **Table 2**).

Discussion

DLBCL is the most commonly NHL among adults, with an annual incidence of 7-8 cases per 100,000 people for each year [11]. Despite the generally favorable prognosis after the initial treatment, approximately 40% DLBCL patients resistant to different therapies [12]. The-refore, it is cri-tical to understand the pathogenesis of the disease and develop no-vel treatments.

B7-H4 is one of the most recently identified members of B7 family of costimulatory molecules serving as a negative regulator of the T cell mediated immune response [5]. Previous studies have demonstrated that B7-H4 is highly expressed in many different types of human cancers and mostly associated with poor clinical outcomes [6]. However, B7-H4 expression in DLBCL has not been studied. In the current study, we for the first time identified elevated expression levels of B7-H4 in DLBCL tissues. B7-H4 was high expressed in 58.03% of DLBCL tissues, significantly higher than the 21.43% in adjacent normal tissues. Our results were consistent with previous reports that B7-H4 was upregulated in numerous hu-man malignancies [13]. For instance, In a study involving 108 cases of cervical cancer conducted by Huang et al, the rate of expression of B7-H4 in tumor tissues was reported to be high (72.22%, 78/108) [14]. In a study involving a large number of patients with prostate cancer, Zang et al reported the abnormally high expression of B7-H4 in tumor tissues with positive expression rates of 99% [15]. Several groups have reported that high B7-H4 levels were assoc--iated with patients' clinical features, including tumor size, lymph node metastasis, and tumor stage [16-18], suggesting an importance role of B7-H4 in the development and progression of cancer. Herein, our data also revealed that B7-H4 expression in DLBCL was correlated with tumor stage and extranodal sites among all the clinicopathologic features evaluated. Furthe-rmore, patients with a high level of B7-H4 expression tended to have a poor prognosis. Additionally, multivariate analysis confirmed that the B7-H4 expression was identified as an independent risk factor for the clinical prognosis of DLBCL. Therefore, B7-H4 might serve as a potential biomarker for predicting tumor progression in patients with DLBCL.

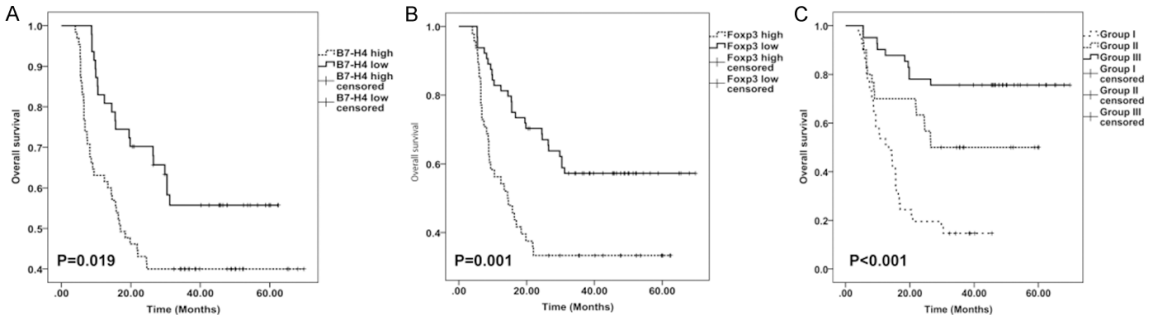

Figure 3. A. Overall survival of 112 DLBCL patients in relation with B7-H4 protein expression, high B7-H4, n=65; low B7-H4, n=47. B. Overall survival of 112 DLBCL patients in relation with Foxp3 protein expression, high Foxp3, n=48; low Foxp3, n=64. C. Based on the B7-H4 and Foxp3 expression levels, 112 patients were divided into three groups: high B7-H4 and Foxp3 (Group I, n=41), high B7-H4 and low Foxp3, or low B7-H4 and high Foxp3 (Group II, n=30), and low B7-H4 and low Foxp3 (Group III, n=41). Overall survival time was calculated using the Kaplan-Meier method and analyzed using the log-rank test.

Table 2. Univariate and multivariate analyses of prognostic factors in patients with DLBCL

Variables	Univariate	Multivariate		
	P value	Hazard Ratio	95% CI	*P value*
Gender (Male *vs.* Female)	0.820	0.921	0.714-1.139	0.920
Age (<60 *vs.* ≥60 years)	0.041	1.237	0.974-1.624	0.312
Stage (I-II vs. III-IV)	0.000	1.579	1.187-2.541	0.013
Pre-treatment LDH (Normal *vs.* Elevated)	0.689	0.910	0.701-1.142	0.362
Extranodal sites (0-1 *vs.* ≥2)	0.537	1.167	0.730-1.321	0.861
IPI score (0-2 *vs.* 2-4)	0.012	1.357	0.815-1.336	0.271
B7-H4 expression (High *vs.* Low)	0.000	1.589	1.247-3.697	0.002
Foxp3 expression (High *vs.* Low)	0.000	2.318	1.974-3.971	0.008

Tumor immune microenvironment can induce immune resistant to tumor cells, and promote tumor cells to escape immune surveillance. Treg cells are a main type of immunosuppressive cells involved in tumor immune escape [19]. The forkhead transcription factor FoxP3 is critically involved in the development and function of Treg cells. Several reports have shown that $Foxp3^+$ Treg cells were increased in the tumor infiltrating lymphocytes and the increased number of $Foxp3^+$ Treg cells in tumor tissues was correlated with poor prognosis [20]. Consistent with previous findings, we also found elevated Foxp3 expression in DLBCL tissues (42.85%, 48/112). Our results also suggest that high expression of Foxp3 is associated with poor prognosis in DLBCL patients and is an independent prognostic predictor of the clinical outcome. In the tumor microenvironment, the expression of B7-H4 was reported to be positively correlated with Tregs infiltration in many tumors, including ovarian cancer [21], gastric cancer [22], cervical cancer [14, 23], and colorectal cancer [24]. B7-H4 can promote the proliferation of Tregs and the secretion of IL-10 and TGF-β1 *in vitro*, suggesting that B7-H4 can promote the immune function of Treg cells [23]. In our study, we found that the expression of B7-H4 in DLBCL tissues was positively correlated with Foxp3 expression (r=0.22, *P*=0.019). In addition, patients with high B7-H4 and Foxp3 expression exhibited a significantly the worst OS compared to patients with high/low B7-H4 and low/high Foxp3, or low B7-H4 and low Foxp3, suggesting B7-H4 may mediate tumor immune escape by increasing the proportion of Treg cells in the tumor microenvironment. Consequently, inhibition of B7-H4 may reduce or block the proliferation of Treg cells, leading to antitumor effects in DLBCL immunotherapy. However, the underlying mechanism that the stimulatory effect of B7-H4 on Tregs infiltration remains unclear.

In summary, our observation showed that B7-H4 correlates with Treg cells and high level

of B7-H4 and Foxp3 is associated with a poor clinical outcome in patients with DLBCL, suggesting that B7-H4 and Foxp3 may be useful as a prognostic factor and for diagnosis of DLBCL. Further studies are needed to investigate the mechanism underlying the inhibitory function of B7-H4 on the prognosis of DLBCL.

Acknowledgements

This work was supported by Health & Medicine and Science & Technology Program of Zhejiang province (No.2016RCB018).

Disclosure of conflict of interest

None.

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