Int J Clin Exp Med 2017;10(8):12361-12366
www.ijcem.com /ISSN:1940-5901/IJCEM0058938

Original Article

The expression of B7-H4 in serum and lymphoma tissues and its clinical significance

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Received June 7, 2017; Accepted June 30, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Objective: To investigate the expression of B7-H4 in serum and lymphoma tissues of patients and its clinical significance. Methods: Ninety-eight lymphoma patients, who were treated in our hospital from January 2014 to December 2016, were recruited and divided into the early-onset group (76 cases; 12 cases with Hodgkin's lymphoma (HL), 64 cases with Non-Hodgkin's lymphoma) and the recurrence group (22 cases). Additionally, the healthy controls consisted of 60 healthy volunteers. Meanwhile, 50 cases of lymphoma tissue specimens with complete information were collected and the controls were 39 cases of reactive lymphoid hyperplasia. The expression levels of B7-H4 in serum and lymphoma tissue were detected by enzyme-linked immunosorbent assay and immunohistochemistry, respectively. Results: The expression level of B7-H4 in serum of lymphoma patients was significantly higher than that of healthy controls ($P<0.01$), however, in which the difference was insignificant between the patients with Non-Hodgkin's lymphoma and patients with Hodgkin's lymphoma ($P>0.05$). In addition, the expression level of B7-H4 in early on set group was evidently lower than that of recurrence group ($P<0.05$). The B7-H4 positive rate in lymphoma tissues was drastically higher than that in reactive lymphoid hyperplasia with significant difference ($P<0.05$), while it showed an opposite result between Hodgkin's lymphoma tissues and Non-Hodgkin's lymphoma tissues ($P>0.05$). Conclusion: B7-H4 was high-expressed in serum and lymphoma tissues. And B7-H4 which is associated with the development and progression of lymphoma, has certain clinical significance for auxiliary diagnosis of lymphoma.

Keywords: B7-H4, lymphoma, non-hodgkin's lymphoma, hodgkin's lymphoma

Introduction

Lymphoma mainly develops in lymph nodes or other lymphatic tissues, which is a kind of hematologic malignancy with high morbidity. At present, little is known about the pathogenesis of lymphoma and there is no effective treatment for it. The B7 families are co-stimulatory molecules that participate in the cell signaling path between antigen-presenting cells and T cells [1-3]. Interestingly, the B7 family can also provide co-inhibitory signals for the inhibition of T cell activation. Therefore, the deficiency and increase of the expression of B7 families as co-stimulatory molecules or co-inhibitory molecules both can help tumors to avoid the monitoring of immune system, thus to escape from the damage by T cells. Hence, it is of great importance for seeking an effective anti-tumor immunity via regulating B7 molecule-related signaling pathways.

B7-H4, one of the known seven members of B7 family, is a negative regulatory molecule in the T cell immune response, which can inhibit proliferation of T cell and these cretion of cytokines *in vivo* [4, 5]. Currently, the expression level of B7-H4 has been significantly up-regulated in many human tumor tissues such as esophageal carcinoma, lung carcinoma, gastric carcinoma and breast carcinoma [6-9]. Additionally, B7-H4 can also detect in the serum of patients with

tumor [10, 11]. So far, however, there are few reports about the expression and the function of B7-H4 in lymphoma. Therefore, this study aimed to detect the expression level of B7-H4 in tissues and serum of lymphoma patients by immunohistochemistry and enzyme-linked immunosorbent assay so as to provide experiment basis and theoretical basis for clinical diagnosis and potential treatment of lymphoma.

Materials and methods

General information

This study was approved by Ethics Committee of our hospital and all the enrolled patients or their families had signed informed consent.

Ninety-eight hospitalized patients with lymphoma who were treated in our hospital from January 2014 to December 2016 were selected as subjects and they were divide into the early-onset group (76 cases) and the recurrence group (22 cases). Among them, the early-onset group contained 47 males and 29 females with ages ranging from 15 to 68 years old, of which 12 cases were Hodgkin's lymphoma (HL) and 64 cases were Non-Hodgkin's lymphoma (NHL) according to their pathologic diagnosis. Meanwhile, 60 healthy volunteers were recruited as healthy controls, including 36 males and 24 females with ages ranging from 18 to 70 years old.

Moreover, 50 lymphoma tissue specimens were collected in the same period from patients including 29 males and 21 females with ages ranging from 21 to 77 years old and among them, there are 28 specimens with Non-Hodgkin's lymphoma and 22 specimens with Hodgkin's lymphoma. The control group consisted of 39 specimens of lymphocyte reactive hyperplasia, from 21 males and 18 females that all aged from 20 to 75 years old.

Main reagents

Human B7-H4 ELISA kit was purchased from Shanghai Westang Biotech Co., Ltd.; rabbit anti-human B7-H4 antibody was purchased from the United States Sigmacompany; DAB color developing reagent and citrate buffer powder were all purchased from Shanghai Xin Yu Biotech Co., Ltd.

ELISA for detecting the expression level of B7-H4

3 mL peripheral blood was collected from all the lymphoma patients and the healthy subjects, and then the serum was separated by centrifugation at 2000 rpm/min for 3 min, and stored at -80°C for the later detection. Detection of the concentration of B7-H4 in serum of patients was carried out in strict accordance with the instruction manual of ELISA kit. Briefly, the standard substance was taken out and equipoised at the room temperature for 5 min. Then the standard substance and samples were added to the reaction plates, respectively, and incubated at 37°C for 2 h. Subsequently, the reaction system was incubated with rabbit anti human B7-H4 primary antibody (1:200 dilution) at 37°C for 1 h followed by washing with PBS for 3 times and incubated with avidin at 37°C for 30 min. After a further 3 washes with PBS, dry the reaction plates, add the chromogenic substrate and end the reaction 15 min later. Then, the OD value was detected at 450 nm. The standard curve was drawn by using the standard concentration as horizontal axis and the A_{450} value as vertical axis to calculate the level of B7-H4 in serum.

Immunohistochemistry for detection the expression of B7-H4 in Lymphoma tissues

Lymphoma tissues were embedded with paraffin and cut into slices, and were dewaxed and dehydrated, then the slices were incubated with 3% H_2O_2 for 10 minutes at room temperature to eliminate the activity of endogenous peroxidase, then blocked by normal 10% goat serum for 15 min after washing with PBS. After shaking off the blocking serum slightly, the slices were incubated with rabbit anti human B7-H4 primary antibody in the wet box at 4°C overnight. Next day, the slices were subsequently washed 3 times with PBS (each time for 5 minutes) and incubated with secondary antibody (horse radish peroxidase labeled goat anti rabbit IgG) for 30 min at 37°C. Later, wash again with PBS with the same frequency as above. After this, the DAB color developing reagent was applied for staining and the optical microscope was used to control the degree of coloration, which were then ended by washing with distilled water when the coloration was complete. Finally, all the slices were counter-

Table 1. The expression of B7-H4 in two kinds of lymphoma patients' serum of primary group and in health controls ($\bar{x}\pm s$)

Groups	Cases	B7-H4 (μg/L)
NHL group	64	37.92±10.41*
HL group	12	39.47±10.57#
Health controls	60	26.19±10.33

Note: *P=0.002, #P=0.001 compared with the health controls.

Table 2. The expression of B7-H4 in lymphoma patients' serum of primary group, recurrent group and health control group ($\bar{x}\pm s$)

Groups	Cases	B7-H4 (μg/L)
Early onset group	76	35.68±10.39*,#
Recurrent group	22	43.04±11.45Δ
Health controls	60	26.19±10.33

Note: *P=0.001, ΔP=0.000 compared with the health controls; #P=0.004 compared with the recurrent group.

stained with hematoxylin and mounted with neutralbalsam.

Results evaluation criteria

It represented the expression of B7-H4 was positive when the staining color in cytoplasm or cytomembrane is yellow and brown, conversely, if not, the expression of B7-H4 was negative. Besides, six high magnification visual fields (400X) were randomly selected, and the positive degree was evaluated by semi quantitative analysis according to the positive cell rate: the positive cell rate <5%, (-); the positive cell rate 5%-10%, (+); the positive cell rate 10%-50%, (++); the positive cell rate 50%-80%, (+++); the positive cell rate >80%, (++++).

Statistical analyses

The data were analyzed by SPSS19.0. The measurement data were expressed as mean ± standard deviation and the comparison between the two groups applied t test, while the comparison between the three groups adopted one-way ANOVA. The count data were expressed as percentage and the comparison between groups applied chi-square test. The comparison of ranked data between groups used the rank sum test. When P<0.05, differences were considered statistically significant.

Results

The expression of B7-H4 in two kinds of lymphoma patients' serum of early onset group

The expression level of B7-H4 in serum of NHL patients was 37.92±10.41 μg/L, which in serum of HL patients was 39.47±10.57 μg/L, and there was not significantly different (P>0.05). But the expression levels of B7-H4 in serum in both HL and NHL patients were obviously higher than that in health controls and the differences has statistical significance. As shown in **Table 1**.

The expression of B7-H4 in lymphoma patients' serum of early onset group, recurrent group and health controls

The expression level of serum B7-H4 in the early onset group was 35.68±10.39 μg/L, which in the recurrent group was reached 43.04±11.45 μg/L and the differences between the two groups was statistically significant (P<0.05). Additionally, the expression level of serum B7-H4 in health control group was 26.19±10.33 μg/L, which was significantly different with that in other two groups (P<0.01). As shown in **Table 2**.

Comparison of the expression of B7-H4 in lymphoma tissues and reactive lymphoid hyperplasia

The results of immunohistochemistry showed that the area with yellow brown color were mainly distributed in cytoplasm and cytomembrane, indicating that the expression of B7-H4 was higher in these two sites and the positive rate of B7-H4 in lymphoma tissues was 48%, while in reactive lymphoid hyperplasia did not see the yellow brown staining color (P<0.05), which showed B7-H4 did not express in reactive lymphoid hyperplasia. As shown in **Table 3**; **Figure 1**.

Furthermore, the positive rate of B7-H4 in lymphoma tissue specimens from NHL patients was 50% (14/28), including 5 cases (+), 5 cases (++), 3 cases (+++) and 1 cases (++++), while which from HL patients was 45.5% (10/22) consisting of 8 cases (+), and 2 cases (++) and there was no significant difference between them (P>0.05). As shown in **Figure 2**; **Table 4**.

Table 3. Comparison of the expression of B7-H4 in lymphoma pathological tissue and reactive lymphoid hyperplasia

Group	Cases	-	+	++	+++	++++	U	P
Lymphoma tissue	50	26	13	7	3	1	2.49	<0.05
Reactive lymphoid hyperplasia	39	39	0	0	0	0		

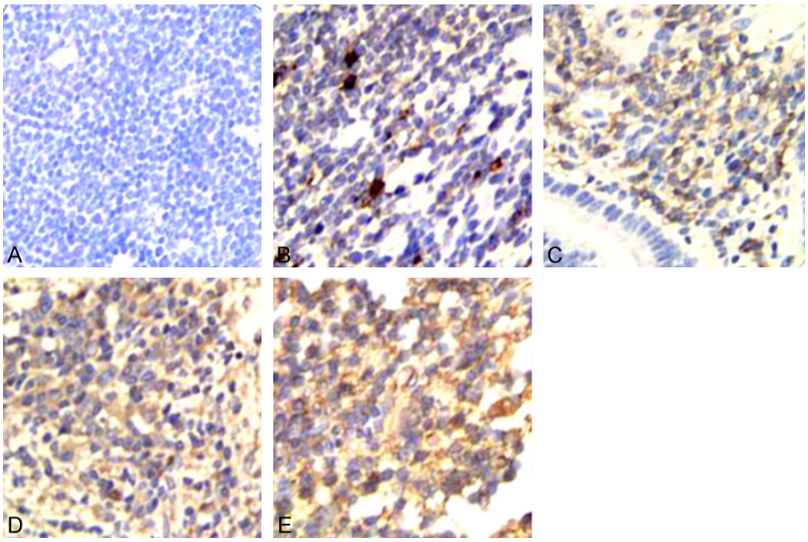

Figure 1. Immunohistochemical staining was used to detect the expression of B7-H4 in lymphoma pathological tissue (400X). A: The expression of B7-H4 in lymphoma tissue was negative; B: The expression of B7-H4 in lymphoma tissue was +; C: The expression of B7-H4 in lymphoma tissue was ++; D: The expression of B7-H4 in lymphoma tissue was +++; E: The expression of B7-H4 in lymphoma tissue was ++++.

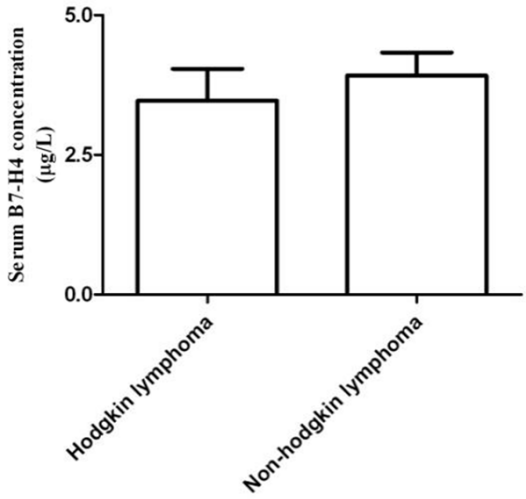

Figure 2. The positive expression rate of B7-H4 in lymphoma tissues.

Discussion

Lymphoma is an immune cell tumor and its etiology is not clear. Most scholars believed that viral infection, radiation, heredity and chemical factors played a role in the pathogenesis of lymphoma. Immunocompromised patients were more likely to develop lymphoma and their incidences was higher than normal population [12]. In spite of the development of medical diagnostic techniques and the advent of immune targeted drugs, such as rituximab, have markedly improved the level of diagnosis and treatment of lymphoma and some types of lymphomas had showed good curative effect, there were still some other pathological types of lymphomas that were difficult to diagnose in clinical practice, meanwhile, they had poor prognosis and didn't have definitive treatment methods. Therefore, an early and timely diagnosis of lymphoma and the search for new targeted therapies have a very vital clinical and practical significance.

B7 family is an important class of co-signaling molecules. As one of them, B7-H4 was considered as a sensitive tumor marker for early diagnosis of tumor *in vivo* and an evaluation index of tumor treatment effect and prognosis. Studies showed that the expression of B7-H4 was significantly increased in various tumor tissues [13-15]. For example, it was overexpressed in gastric cancer and pancreatic cancer and had positive correlation with the clinical stage of patients [16, 17]. In addition, many studies showed that the expression of B7-H4 was closely related to the typing and staging of tumor and patients' prognosis [18, 19]. However, there were few reports about the role of B7-H4 in the pathogenesis and development of lymphoma. As B7-H4 could inhibit the cell proliferation of T cells, the secretion of cytokines and the process of cell cycle, it also negatively controlled the T cell-mediated immune response [20, 21]. Thereby, B7-H4 might play a

Table 4. The expression levels of B7-H4 in lymphoma tissue specimens from NHL patients and HL patients

Group	Cases	-	+	++	+++	++++	U	P
NHL tissues	28	14	5	5	3	1	1.08	>0.05
HL tissues	22	12	8	2	0	0		

key role in the effects of the organism against tumors by the T cell-mediated immune response, thus we suspect that there may be a close relationship between B7-H4 and the occurrence and development of lymphoma.

At present, the synthesis of B7-H4 and its mechanism in tumor were still unclear. Therefore, in this study, the expression level of B7-H4 in serum and lymphoma tissues of patients was detected via enzyme-linked immunosorbent assay and immunohistochemistry. The former results showed that the expression level of B7-H4 in serum of lymphoma patients was significantly higher than that in healthy controls ($P<0.01$), similarly, the expression level of serum B7-H4 in the early onset group was significantly lower than that in the recurrent group ($P<0.01$), indicating that the expression level of B7-H4 in patients' serum could be used as one of the tumor markers for lymphoma recurrence. On the other hand, the results of immunohistochemistry showed that the positive rate of B7-H4 in lymphoma tissues was significantly higher than that in reactive lymphoid hyperplasia ($P<0.05$), showing that B7-H4 could be applied as an effective marker to distinguish lymphoma and reactive lymphoid hyperplasia. Additionally, there was no evident difference in the expression of B7-H4 in serum and pathological tissues between patients with NHL or HL ($P>0.05$), manifesting that there was no clinical significance for B7-H4 working as a differential diagnosis to NHL and HL.

This study confirmed the high expression level of B7-H4 in the serum and lymphoma tissues of patients with the aim to provide the experimental and theoretical basis for the aided diagnosis of lymphoma and the development of its targeted drugs. However, B7-H4 still needs to be confirmed that whether it can apply as a new tumor marker in clinical treatment by further studies. In addition, as B7-H4 is produced by tumor cells, its specific production and mechanism need to be further studied.

Disclosure of conflict of interest

None.

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References

[1] Greenwald RJ, Freeman GJ and Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005; 23: 515-548.

[2] Xu Z, Shen J, Wang MH, Yi T, Yu Y, Zhu Y, Chen B, Chen J, Li L, Li M, Zuo J, Jiang H, Zhou D, Luan J and Xiao Z. Comprehensive molecular profiling of the B7 family of immune-regulatory ligands in breast cancer. Oncoimmunology 2016; 5: e1207841.

[3] Cheng H, Janakiram M, Borczuk A, Lin J, Qiu W, Liu H, Chinai JM, Halmos B, Perez-Soler R and Zang X. HHLA2, a new immune checkpoint member of the B7 family, is widely expressed in human lung cancer and associated with EGFR mutational status. Clin Cancer Res 2017; 23: 825-832.

[4] Podojil JR and Miller SD. Potential targeting of B7-H4 for the treatment of cancer. Immunol Rev 2017; 276: 40-51.

[5] Iizuka A, Kondou R, Nonomura C, Ashizawa T, Ohshima K, Kusuhara M, Isaka M, Ohde Y, Yamaguchi K and Akiyama Y. Unstable B7-H4 cell surface expression and T-cell redirection as a means of cancer therapy. Oncol Rep 2016; 36: 2625-2632.

[6] Wang L, Cao NN, Wang S, Man HW, Li PF and Shan BE. Roles of coinhibitory molecules B7-H3 and B7-H4 in esophageal squamous cell carcinoma. Tumour Biol 2016; 37: 2961-2971.

[7] Schalper KA, Carvajal-Hausdorf D, McLaughlin J, Altan M, Velcheti V, Gaule P, Sanmamed MF, Chen L, Herbst RS and Rimm DL. Differential expression and significance of PD-L1, IDO-1, and B7-H4 in human lung cancer. Clin Cancer Res 2017; 23: 370-378.

[8] Geng Y, Wang H, Lu C, Li Q, Xu B, Jiang J and Wu C. Expression of costimulatory molecules B7-H1, B7-H4 and Foxp3+ Tregs in gastric cancer and its clinical significance. Int J Clin Oncol 2015; 20: 273-281.

[9] Tringler B, Zhuo S, Pilkington G, Torkko KC, Singh M, Lucia MS, Heinz DE, Papkoff J and

Shroyer KR. B7-h4 is highly expressed in ductal and lobular breast cancer. Clin Cancer Res 2005; 11: 1842-1848.
[10] Zhang SA, Wu ZX, Zhang X, Zeng ZY and Li DL. Circulating B7-H4 in serum predicts prognosis in patients with hepatocellular carcinoma. Genet Mol Res 2015; 14: 13041-13048.
[11] Xu C, Qian L, Yu L, Zhang X and Wang Q. Evaluation of serum and pleural levels of soluble B7-H4 in lung cancer patients with pleural effusion. Biomarkers 2015; 20: 271-274.
[12] Ponce RA, Gelzleichter T, Haggerty HG, Heidel S, Holdren MS, Lebrec H, Mellon RD and Pallardy M. Immunomodulation and lymphoma in humans. J Immunotoxicol 2014; 11: 1-12.
[13] Wang L, Heng X, Lu Y, Cai Z, Yi Q and Che F. Could B7-H4 serve as a target to activate anticancer immunity? Int Immunopharmacol 2016; 38: 97-103.
[14] Sun Y, Wang Y, Zhao J, Gu M, Giscombe R, Lefvert AK and Wang X. B7-H3 and B7-H4 expression in non-small-cell lung cancer. Lung Cancer 2006; 53: 143-151.
[15] Wang X, Wang T, Xu M, Xiao L, Luo Y, Huang W, Zhang Y and Geng W. B7-H4 overexpression impairs the immune response of T cells in human cervical carcinomas. Hum Immunol 2014; 75: 1203-1209.
[16] Arigami T, Uenosono Y, Ishigami S, Hagihara T, Haraguchi N and Natsugoe S. Clinical significance of the B7-H4 coregulatory molecule as a novel prognostic marker in gastric cancer. World J Surg 2011; 35: 2051-2057.
[17] Xu H, Chen X, Tao M, Chen K, Chen C, Xu G, Li W, Yuan S and Mao Y. B7-H3 and B7-H4 are independent predictors of a poor prognosis in patients with pancreatic cancer. Oncol Lett 2016; 11: 1841-1846.
[18] Jiang J, Zhu Y, Wu C, Shen Y, Wei W, Chen L, Zheng X, Sun J, Lu B and Zhang X. Tumor expression of B7-H4 predicts poor survival of patients suffering from gastric cancer. Cancer Immunol Immunother 2010; 59: 1707-1714.
[19] He C, Qiao H, Jiang H and Sun X. The inhibitory role of b7-h4 in antitumor immunity: association with cancer progression and survival. Clin Dev Immunol 2011; 2011: 695834.
[20] Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, Chapoval AI, Flies DB, Bajorath J and Chen L. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity 2003; 18: 849-861.
[21] Choi IH, Zhu G, Sica GL, Strome SE, Cheville JC, Lau JS, Zhu Y, Flies DB, Tamada K and Chen L. Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. J Immunol 2003; 171: 4650-4654.