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Expression of co-inhibitory molecules B7-H4 and B7-H1 in Epstein-Barr virus positive diffuse large B-cell lymphoma and their roles in tumor invasion

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ABSTRACT

To investigate the relationship between immunoregulatory molecules B7-H4 and B7-H1 in Epstein-Barr positive diffuse large B-cell lymphoma (EBV$^+$DLBCL). Immunohistochemistry was used to detect the expression of B7-H4 and B7-H1 in tumor tissues of 13 patients with EBV$^+$DLBCL. The expression levels of B7-H4 and B7-H1 in four diffuse large B-cell lymphoma cell lines (SU-DHL-4, SU-DHL-10, SU-DHL-6, Pfeiffer) were analyzed by flow cytometry. Transwell invasion assays were conducted to observe the invasive ability of cell lines. B7-H4 and B7-H1 were expressed in 84.62% and 100% tumor specimens of EBV$^+$DLBCL. The overexpression of B7-H4 and B7-H1 was found in 46.15% and 23.08% tumor samples of EBV$^+$DLBCL. There was a medium negative correlation between the expression levels of B7-H4 and B7-H1 ($r = -0.667$, $P = 0.013$, spearman rank correlation). The expression levels of B7-H1 in four diffuse large B-cell lymphoma cell lines were positively correlated with their invasive ability, whereas the expression levels of B7-H4 were not. Here, we provide evidence for the negative relationship between B7-H4 and B7-H1 in EBV$^+$DLBCL. The expression of B7-H1 in EBV$^+$DLBCL appears to be the dominant factor which affects tumor aggressiveness. When B7-H1 expression weakens, the molecule B7-H4 may become the dominant factor of prognosis in patients with EBV$^+$DLBCL.

1. Introduction

Epstein-Barr virus positive diffuse large B-cell lymphoma (EBV$^+$DLBCL) accounts for 8–10% of diffuse large B-cell lymphoma (DLBCL), which always occurs in elderly immunocompromised patients and is usually associated with high malignancy and poor clinical outcome [1,2].

The role of negative immune regulatory molecules in lymphoma is becoming a hot topic in recent years. NCI immunotherapy Agent Workshop has managed to find out the most potential candidates for negative immune regulatory targets in cancer immunotherapy from 2007. Programmed death receptor-1/ Programmed death ligand 1 (PD-1/PD-L1) and B7-H4 are the key targets of T cell negative regulatory factors in their list [3].

Kiyasu has shown that the overall survival of PD-L1 (B7-H1) positive diffuse large B-cell lymphoma is shorter than that of PD-L1 (B7-H1) negative DLBCL [4]. The PD-1/PD-L1 (B7-H1) signaling pathway triggers tumor cells escape from immune surveillance, thus the therapeutic antibodies targeting the PD-1/PD-L1 (B7-H1) signal pathway is one of the promising methods for patients with relapsed and refractory diffuse large B-cell lymphoma. Especially, PD-1 monoclonal antibodies have been proved to be active in primary mediastinal and central nervous system (CNS) large B-cell lymphoma [5,6].

Although PD-1/PD-L1 inhibitor is a breakthrough for the therapy of lymphoma, some patients are still unlikely to benefit from PD-1/PD-L1 targeted immunotherapy. Are there other negative immune regulatory molecules involved in the antibody-resistant mechanism? What is the role of another important co-inhibitory B7-H4 molecule in EB virus-associated diffuse large B-cell lymphoma? To address this question, we have previously detected the expression of B7-H4 in EBV$^+$DLBCL and

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its role in lymphoma. We have found that B7-H4 inhibits apoptosis in EBV-infected DLBCL cell line via the Erk1/2 and Akt pathways. Therefore, it is speculated that B7-H4 may play a key role in the development of EBV$^+$DLBCL [7].

Then the next question has come up to us what is the relationship of these two crucial molecules B7-H1 and B7-H4 in EBV$^+$DLBCL. Which molecule acts as the dominant factor in development of EBV$^+$DLBCL? How about the therapeutic value of B7-H4 inhibitor? Therefore, this study was conducted to explore the expression of B7-H4 and B7-H1 in tumor tissues from patients with EBV-positive diffuse large B-cell lymphoma in vivo and the invasive ability of diffuse large B-cell lymphoma cell lines in vitro.

2. Materials and methods

2.1. Tissue species and cell lines

Thirteen tumor specimens of Epstein-Barr virus-positive diffuse large B-cell lymphoma patients at the Department of Hematology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine were analyzed after the presence of adequate amounts of tumor were confirmed by representative hematoxylin and eosin (H&E)-stained slides for histological examination. Four diffuse large B-cell lymphoma cell lines (SU-DHL-4, SU-DHL-10, SU-DHL-6, Pfeiffer) were purchased from ATCC. Mouse monoclonal antibody (3E8) against human B7-H4 is a gift from Institute of Infectious Diseases affiliated Zhejiang University. The present study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine affiliated Shanghai General Hospital (No. 2019KY018).

2.2. Flow cytometry

Flow cytometric analysis of four diffuse large B-cell lymphoma cell lines (SU-DHL-4, SU-DHL-10, SU-DHL-6, Pfeiffer) was performed to detect the expression of B7-H4 and B7-H1 in these cell lines. Each of the four cell lines was divided into four tubes ($1.0*10^6$ cells and 100 μl/tube) which named as experimental group, B7-H1 isotype control groups, B7-H4 isotype control groups and blank group according to the different antibodies. In the experimental group, 5 μl B7-H4-APC (BD) and 5 μl B7-H1-Pecy7 (Biolegend) were added to each tube. B7-H1 isotype group was identified after 5 μl B7-H1-Pecy7 (Biolegend) and 5 μl Mouse IgG1 kappa Isotype control-APC (invitrogen)were added. Conversely, the B7-H4 isotype group contained 5 μl B7-H4-APC (BD) and 5 μl Mouse IgG1 kappa Isotype Control-Pecy7 (Biolegend). Cells were incubated without any antibody in blank group. Finally, the suspension cells were detected by the Life Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).

2.3. Cell culture

According to the relevant instructions of ATCC, the culture medium of SU-DHL-4 is IMDM + 10% FBS and the medium of SU-DHL-10, SU-DHL-6 and Pfeiffer is RPMI 1640 + 10% FBS. The culture temperature maintained at 37℃ and the culture media were replaced every 2–3 days depending on the cells status afterwards.

2.4. Cell invasion assay

The invasive assay was tested using the kit of the 6.5 mm diameter membrane with 8 μm pore size (corning) and BD Matrigel™ Basement Membrane Matirx (BD Biocoat). SU-DHL-4, SU-DHL-10, SU-DHL-6 and Pfeiffer cells ($2*10^6$ cells) were seeded in the upper part of transwell plate for 48 h incubation respectively. Then, the suspension cells in the lower chamber were photographed under a light microscope and counted by Blood Cell Count Board. Because scanty cells of SU-DHL-6 and Pfeiffer in the lower reservoir were harvested at 48 h, the incubation time of these two cell lines was appropriately extended to 96 h for quantifying. The experiments of cell lines were repeated for three times.

2.5. Immunohistochemistry staining

Immunohistochemistry (IHC) staining was carried out using 3E8 monoclonal antibody (mAb) as the primary antibody followed by reagents from the EnVision System (DAKO, CA, USA) [8]. The specimens were subjected to antigen retrieval and incubated with anti-PD-L1 antibody (28-8) (Abcam, Cambridge, MA) for 60 min at room temperature. The rabbit specific IHC polymer detection kit HRP/DAB (Abcam, Cambridge, MA) was used to detect the antibodies bound to antigens within specimens by light microscopy. Semi-quantitative measurements of staining intensity (0–3, i.e., least intense to most intense) and the proportion of stained cells (0–4, i.e., no cells stained to more than 75% cells stained) were determined as previously described [8]. In this study, expression of B7-H4 and B7-H1 was considered as expression when combined scores ≥ 2 and overexpression when combined scores ≥ 6 [8].

2.6. Statistical analysis

Statistical analysis was performed with SPSS 23.0 software. Correlation analysis was conducted by Pearson or Spearman's rank correlation test and Chi-square test was used to compare the differences of positive expression rate. Data is considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Clinical information of patients with Epstein-Barr virus-positive diffuse large B-cell lymphoma

Adequate amounts of tumor specimens determined by IHC were obtained from 13 EBV$^+$DLBCL patients at the Department of Hematology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine whose clinical characteristics are shown in Table 1. The median overall survival time of them was 99 days (15–1385 days).

Table 1
Clinical characteristics of 13 patients with EBV$^+$DLBCL.

Characteristics	No.	%
Sex(male/female)	13(8/5)	
Age(y) median(range)	59(44–84)	
Overall survival time(day)	99(15–1385)	
Initial Chemotherapy		
R-CHOP	6/13	46.15
CHOP	4/13	30.77
MTX + IDA + Dx	1/13	7.69
no treatment	2/13	15.38
Therapeutic response		
Complete remission	2/13	15.38
Incomplete remission	8/13	61.54
loss of follow up	3/13	23.08
Clinical stage		
I	1/12	8.33
II	1/12	8.33
III	0/12	0.00
IV	10/12	83.33
B symptoms present	7/13	53.85
Outcome		
survival	5/13	38.46
death	4/13	30.77
loss of follow up	4/13	30.77

CR, complete remission; IR, incomplete remission; R, rituximab; MTX, Methotrexate; IDA, Idarubicin; Dx, Dexamethasone.

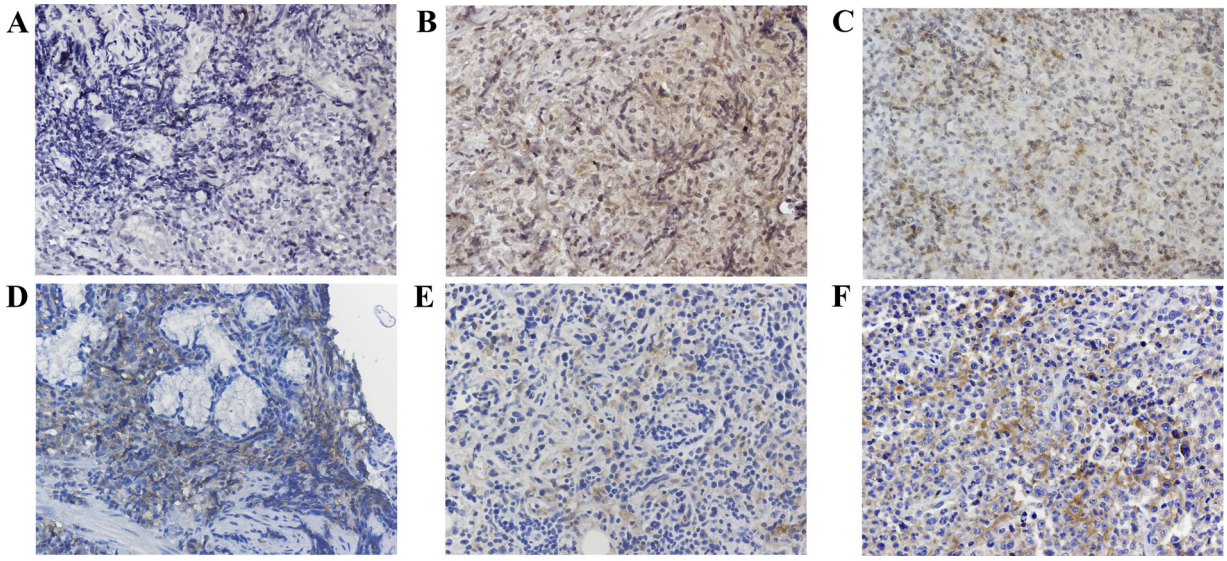

Fig. 1. Immunohistochemical staining results of B7-H4 and B7-H1 in three selected tumor samples from EBV$^+$DLBCL patients. The samples from EBV$^+$DLBCL patients were subjected to IHC staining using B7-H4 antibody 3E8 and anti-PD-L1 antibody (28-8). A–C. Immunohistochemical staining results of B7-H4 in tumor tissues of the three selected patients whose scores were 0, 5 and 3. D–F. Immunohistochemical staining results of B7-H1 in tumor tissues of the three selected patients whose were scored at 6, 2 and 5.

3.2. Expression of B7-H4 and B7-H1 in tumor specimens of patients with EBV$^+$DLBCL

To evaluate the expression of B7-H4 and B7-H1, we detected B7-H4 and B7-H1 in tumor specimens of 13 EBV$^+$DLBCL patients by immunohistochemistry staining (Fig. 1). We found that the expression rate of B7-H4 (score ≥ 2) was 84.62% and the B7-H1 expression rate (score ≥ 2) was 100%. Furthermore, we discovered the overexpression rates of B7-H4 and B7-H1 (score ≥ 6) were only 46.15% and 23.08% (Table 2).

3.3. Negative correlation between the expression levels of B7-H4 and B7-H1 in tumor specimens of patients with EBV$^+$DLBCL

To analyze the correlation between B7-H4 and B7-H1, we performed statistical analysis to seek the relationship between the expression levels of B7-H4 and B7-H1 in tumor samples of patients with EBV$^+$DLBCL. We found that the expression rate of B7-H4 was significantly lower than that of B7-H1 (84.62% vs 100%, P = 0.000, chi-square test). The overexpression rate of B7-H4 was, however, significantly higher than that of B7-H1 (46.15% vs 23.08%, P = 0.001, chi-square test) (Fig. 2). Consequently, the expression levels of B7-H4 and B7-H1 showed a medium-intensity negative correlation (r = −0.667, P = 0.013, spearman rank correlation).

3.4. Strong invasive ability was showed in diffuse large B-cell lymphoma cell line with high expression of B7-H1

In order to clarify the relationship between the expression levels of B7-H4 and B7-H1 and the invasive ability of diffuse large B-cell lymphoma, we measured the B7-H4 and B7-H1 expressed in selected four diffuse large B-cell lymphoma cell lines (SU-DHL-4, SU-DHL-10, SU-DHL-6 and Pfeiffer) and performed cell invasion assay. Flow cytometry analysis was conducted to identify the B7-H4 and B7-H1 expression of four lymphoma cell lines. The results showed that SU-DHL-4 weakly expressed B7-H4 but highly expressed B7-H1. All of the other three cell lines (SU-DHL-10, SU-DHL-6, Pfeiffer) expressed B7-H4 alone but not B7-H1 (Fig. 3). And then cell invasion assay was used to test the invasive abilities of four diffuse large B-cell lymphoma cell lines. According to the number of cells in the lower chamber, the harvest time of SU-DHL-4 and SU-DHL-10 was 48 h and SU-DHL-6 and Pfeiffer cells was appropriately extended to 96 h for quantifying. After $2*10^6$ cells of each cell line were seeded in the upper part of transwell plate for incubation, the proportions of cell lines (SU-DHL-4, SU-DHL-10, SU-DHL-6 and Pfeiffer) that migrated to the lower chamber through these invasive assays were 33.25%, 1%, 0.26% and 0.13%, respectively (Fig. 4). The B7-H1 expression rate of DLBCL cell lines was strongly associated with their proportion of invasive cells (r = 0.999, P = 0.001, Pearson correlation test). However, there was no correlation between the B7-H4 expression rate of DLBCL cell lines and their proportion of invasive cells (r = −0.6, P = 0.4, Pearson correlation test).

Table 2
Expression of B7-H4 and B7-H1 in EBV$^+$DLBCL patients.

Expression	classification	score	No	%
B7-H4	Negative	< 2	2	15.38
	Positive	≥2	11	84.62
	Overexpression	≥6	6	46.15
B7-H1	Negative	< 2	0	0.00
	Positive	≥2	13	100.00
	Overexpression	≥6	3	23.08

4. Discussion

Epstein-Barr virus positive diffuse large B-cell lymphoma is a relatively rare disease. It has more aggressive feature and is associated with poor prognosis. In 2008, World Health Organization (WHO) classified it as “Epstein–Barr virus (EBV)-positive DLBCL of the elderly”. Later, the disease is named as "EBV-positive diffuse large B-cell lymphoma, Non Otherwise Specific" according to the 2016 WHO classification [9]. The overall survival time of 13 EBV$^+$DLBCL patients in this study was only 99 days (15–1385 days) which is consistent with previous studies. Morales has described that 11 cases (15%) with ISH-EBER positive were associated with poor prognosis. The median overall survival (OS) time of patients with EBV$^+$DLBCL was 7 months while that of patients with EBV$^-$DLBCL was 47 months [10]. It has also been found in many other reports that patients with EBV$^+$DLBCL had inferior overall survival (OS) compared with that in patients with EBV$^-$DLBCL [11,12].

Published data have shown that B7-H1(PD-L1) was associated with

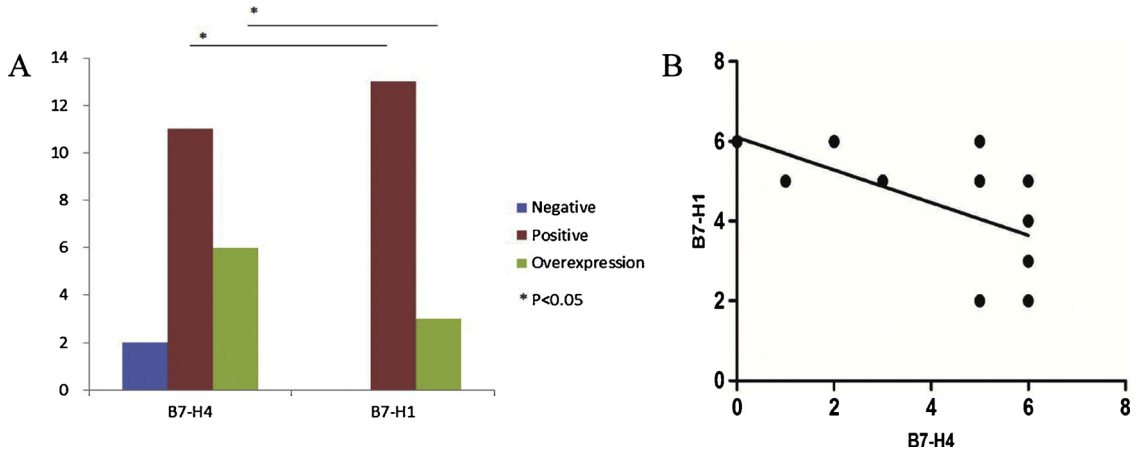

Fig. 2. Expressions of B7-H4 and B7-H1 in EBV$^+$DLBCL patients. A. The number of negative, positive, and overexpressed cases of B7-H4 and B7-H1 in EBV$^+$DLBCL patients. B. B7-H4 and B7-H1 were negatively correlated with moderate intensity in EBV$^+$DLBCL patients ($r = -0.667$, $P = 0.013$, spearman rank correlation). X and Y axes mean the semi-quantitative scores of B7-H4 and B7-H1 in EBV$^+$DLBCL patients.

adverse histopathologic features in various tumors, such as non-clear cell renal cell carcinoma and endometrial tumors [13,14]. Moreover, the study in gastric cancer has identified B7-H1 as a prognostic marker [15]. The overall survival (OS) of patients with B7-H1 positive diffuse large B-cell lymphoma has been significantly shorter than that of patients with B7-H1 negative DLCBL($P = 0.0009$) [4]. Although the programmed death receptor inhibitors are less effective treatment for the DLBCL patients (CR rate 13–18%) in many phase I studies with immunotherapy targeting PD-1/PD-L1(B7-H1) axis [16], they are more effective in patients with primary mediastinal and primary central nervous system B-cell lymphoma whose overall response rates can reach 44% and 100% [5,6]. In addition, as early as 2005 Ichikawa has proposed that tumor cells expressing B7-H4 may help them to escape immune attacks [17]. Subsequent researches have suggested that patients with high B7-H4 expression have poor prognosis [18,19]. Taken together, the expressions of co-inhibitory molecules B7-H4 and PD-1/PD-L1 (B7-H1) in lymphoma are the most promising T-cell negatively co-stimulatory molecules as potential targets for therapeutic intervention [3]. However, little is known about the role of these molecules in patients with EBV$^+$DLBCL.

Some studies have revealed the expression rate of B7-H1 in tumor samples of EBV$^+$DLBCL is 40%, and others have even raised to 100% [20,21]. Nevertheless, the low B7-H1 expression in specimens of the overall diffuse large B-cell lymphoma is confirmed. An analysis of 1253 biopsy samples from DLBCL patients has proved that only 11% of them were B7-H1 positive [4]. Even if PD-L1 (B7-H1) positive and PD-L2 positive specimens of DLBCL are counted together, the positive rate is still only 20–25% [22]. We guess that the different effects of PD-1 antibody on different subtypes of diffuse large B-cell lymphoma may be attributed to different B7-H1 expression in tumor specimens. We found the expression rate of B7-H1 was 100% in tumor specimens of 13 EBV$^+$DLBCL patients. This result revealed that B7-H1 expression in EBV$^+$DLBCL specimens is much higher than that reported in overall DLBCL samples, which is consistent with other researches about EBV$^+$DLBCL [21]. In addition, we also found that the expression rate of B7-H1 was significantly higher than that of B7-H4 in EBV$^+$DLBCL specimens (100% vs 84.62%, $P = 0.000$, chi-square test). Therefore, B7-H1 is highly expressed in tumor tissues of EBV$^+$DLBCL patients which may be a dominant factor contributing to pathogenesis and prognosis of this disease.

PD-1/PD-L1 (B7-H1) signaling is involved in tumor escaping from T cell immune surveillance, thereby resulting in tumor invasion and metastasis [23]. Is there any other mechanism for PD-1/PD-L1(B7-H1) in tumor pathogenesis? B7-H1 levels positively correlates with VEGF ($r = 0.314$, $p = 0.011$) and Ki-67 ($r = 0.391$, $p = 0.001$) levels in gliomas patients which suggest that B7-H1 is important for angiogenesis and proliferation [24]. A direct association between tumor cell proliferation and the B7-H1 expression has also been found in breast cancer patients [25]. Similarly, the association of B7-H1 expression with p-AKT expression in DLBCL ($r = 0.244$, $P = 0.017$) also shows that PD-1/PD-L1 might activate the intracellular AKT/mTOR oncogenic signaling pathway in tumor cells to promote DLBCL aggressiveness [26]. In this study of four diffuse large B-cell lymphoma cell lines, we identified that DLBCL cell lines with high B7-H1 expression has strong invasive ability ($r = 0.999$, $P = 0.001$, Pearson correlation test). Therefore, we concluded that B7-H1 enhanced the invasive ability of diffuse large B-cell lymphoma cell lines.

Many studies showed that limited efficacy of PD-L1 (B7-H1) monoclonal antibody was observed. The findings in patients with meningioma suggested the overall applicability of PD-L1 (B7-H1) monoclonal antibody might be limited [27]. However, our data suggested that B7-H1 could enhance the invasive ability of diffuse large B-cell lymphoma cell lines. The difference between them makes us speculate that other member of B7 family may exhibit a synergistic or antagonistic interaction with the immune checkpoint PD-1 and its receptor B7-H1 (PD-L1). The studies of renal cell carcinoma and colorectal cancer have showed that co-expression of B7-H4 and B7-H1 was associated with poor prognosis in these tumors [28,29]. However, the relationship of B7-H4 and B7-H1 expressions in Epstein-Barr virus-positive diffuse large B-cell lymphoma has not been reported. We examined the expression levels of B7-H4 and B7-H1 in tumor samples of 13 patients with EBV$^+$DLBCL and found that the expression rate of B7-H4 was lower than that of B7-H1. The overexpression rate of B7-H4 was, however, significantly higher than that of B7-H1. In addition, the expression levels of B7-H4 and B7-H1 in tumor specimens of patients with EBV$^+$DLBCL were moderately correlated ($r = -0.667$, $P = 0.013$, spearman rank correlation). Thus, although both B7-H4 and B7-H1 are co-inhibitory molecules, there is a negative correlation between their expressions in the tumor samples of EBV$^+$DLBCL patients. This result is inconsistent with the positive correlation between B7-H4 and B7-H1 found in endometrial tumors [14]. The discrepancy may be due to the difference between these two types of disease. It was confirmed that B7-H4 could inhibit the apoptosis of EBV-infected cell line (Pfeiffer: B7-H4 positive and B7-H1 negative) in our previous study (data are not showed). Therefore, when B7-H1 expression weakens, the mutually exclusive molecule B7-H4 may become the dominant factor of prognosis in patients with EBV$^+$DLBCL. Taken together, we thought that it should be better to try the therapy targeting B7-H4 when anti-PD-1 treatment is ineffective.

The limitation of this study is due to the low incidence of

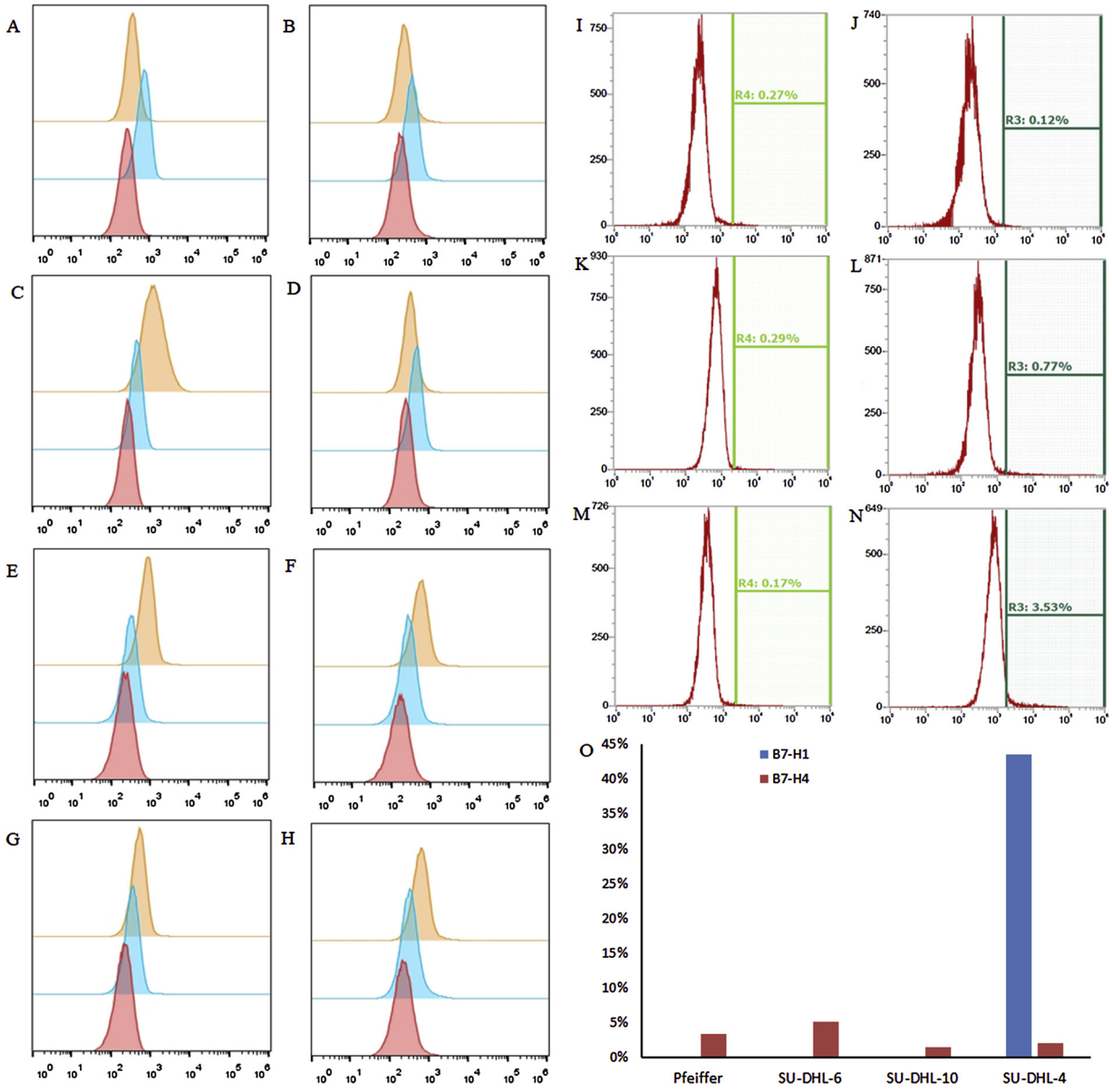

Fig. 3. B7-H4 and B7-H1 expressions in four diffuse large B-cell lymphoma cell lines examined by flow cytometry. The ordinate represents the number of cells, and the abscissa is the fluorescence intensity of B7-H1 or B7-H4. The width of the peak represents the CV (coefficient of variation). A–H. The red peak represents the Blank group (without antibody); the blue peak represents the ISO group (B7-H4 antibody + B7-H1 isotype control antibody or B7-H1 antibody + B7-H4 isotype control antibody); the yellow peak represents the experimental group (B7-H1 antibody and B7-H4 antibody). A–D. Expression of B7-H1 in four diffuse large B-cell lymphoma cell lines in the Blank group, ISO group and experimental group. E–H. Expression of B7-H4 in four diffuse large B-cell lymphoma cell lines in the Blank group, ISO group and experimental group. A and E. Pfeiffer cell line. B and F. SU-DHL-6 cell line. C and G. SU-DHL-4 cell line. D and H. SU-DHL-10 cell line. The synthesized histogram in A is a composite of the histograms in I (Blank group), K (ISO group) and M (experimental group). The histograms in J (Blank group), L (ISO group) and N (experimental group) merge into one in E. O. The bar chart of B7-H4 and B7-H1 expressions in four diffuse large B-cell lymphoma cell lines examined by flow cytometry. The blue bar represents B7-H1 and the red bar represents B7-H4. SU-DHL-4 weakly expressed B7-H4 but highly expressed B7-H1. All of the other three cell lines (SU-DHL-10, SU-DHL-6, Pfeiffer) expressed B7-H4 alone but not B7-H1.

EBV$^+$DLBCL. Further research needs to be conducted among many other EBV$^+$DLBCL patients and explore the downstream signaling pathways and cytokines of B7-H4 and B7-H1 in order to explain the negative relationship between them.

In summary, this is the first report describing the negative relationship between the expression of B7-H4 and B7-H1 in specimens of EBV$^+$DLBCL patients. The expression of B7-H1 in EBV$^+$DLBCL appears to be the dominant factor which affects tumor aggressiveness. When B7-H1 expression weakens, the molecule B7-H4 may become the dominant factor of prognosis in patients with EBV$^+$DLBCL. Thus, this research provides a theoretical basis for combination therapy in EBV$^+$DLBCL patients.

Authors' contribution

YJ was responsible for the study design and the acquisition of data, undertook data analysis, performed the experiments and wrote this paper. JL and JZ performed the Immunohistochemistry (IHC) staining and analyzed the results. SL helped to interpret the data. CW helped to design the experiments and interpret the data. YT undertook project design, data analysis and manuscript revisions.

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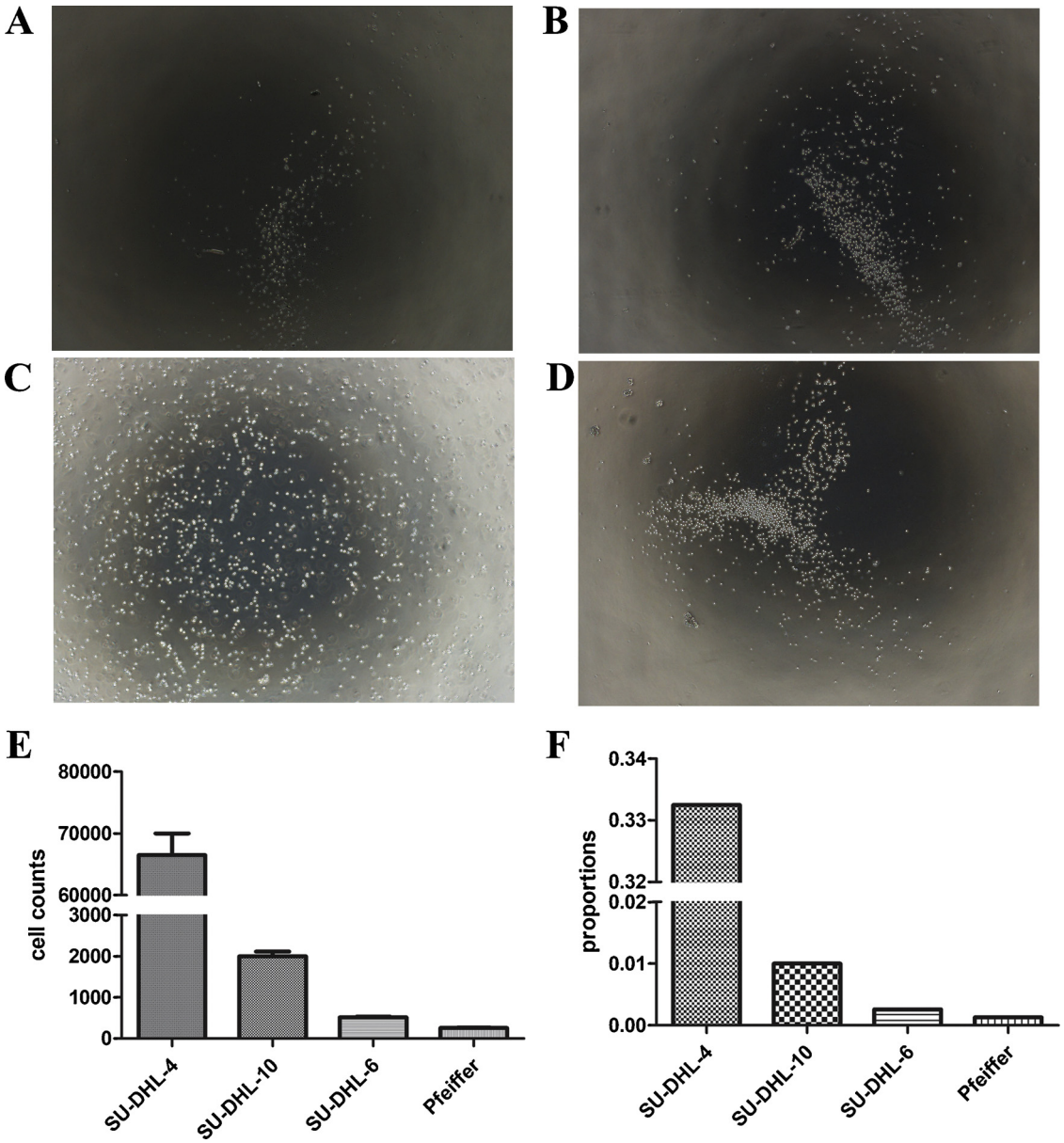

Fig. 4. SU-DHL-4 has the strongest invasive ability among the four diffuse large B-cell lymphoma cell lines. SU-DHL-4, SU-DHL-10, SU-DHL-6 and Pfeiffer cells($2*10^6$ cells) were seeded in the upper part of transwell plate for 48 h incubation respectively. A. Cell photograph of the lower chamber performed by transwell invasion assays of Pfeiffer cells after 96 h of incubation. B. Cell photograph of the lower chamber performed by transwell invasion assays of SU-DHL-6 cells after 96 h of incubation. C. Cell photograph of the lower chamber performed by transwell invasion assays of SU-DHL-4 cells after 48 h of incubation. D. Cell photograph of the lower chamber performed by transwell invasion assays of SU-DHL-10 cells after 48 h of incubation. E. Cell counts of four diffuse large B-cell lymphoma cell lines migrated to the lower chamber through invasive assays. F. Proportions of four diffuse large B-cell lymphoma cell lines migrated to the lower chamber through invasive assays.

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Declaration of Competing Interest

The authors declare that they no financial conflicts of interest.

Acknowledgement

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