RESEARCH ARTICLE

T-cell infiltration and expressions of T lymphocyte co-inhibitory B7-H1 and B7-H4 molecules among colorectal cancer patients in northeast China's Heilongjiang province

Ming Liang · Jingyuan Li · Dandan Wang · Shuchen Li · Yi Sun · Tiewei Sun · Jing Zhang · Xiaoning Chen · Qiang Li · Shibo Sun

Abstract Colorectal cancer has an extremely poor prognosis due to its high rate of recurrence and metastasis. The present study aimed to investigate the correlations between the B7-H1 and B7-H4 expressions as well as the clinicopathological characteristics and the prognosis of patients with colorectal cancer. We further inferred from these findings whether T lymphocyte co-inhibitory molecules (B7-H1 and B7-H4) led to a poor prognosis in Heilongjiang patients with colorectal cancer. Survival analysis revealed that the poor prognosis of these patients was unrelated to patient age, tumor size or histological grade, or lymph node metastasis, but was associated with TNM stage, high B7-H1 and B7-H4 expression levels. High B7-H1 and B7-H4 expressions were closely correlated with poor prognosis in patients with colorectal cancer. We speculate that the joint detection of these molecules may clinically apply for diagnosing and predicting poor prognosis of patients with colorectal cancer in northeast China's Heilongjiang province. In addition, intervention of B7-H1 and B7-H4 may be beneficial for enhancement of immunity in these patients.

Keywords Colorectal cancer · B7-H1 · B7-H4 · Prognosis

Ming Liang, Jingyuan Li, and Dandan Wang contributed equally to this paper.

M. Liang · J. Li · S. Li
Department of Infectious Diseases, The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China

D. Wang
Wu Lian De Memorial Hospital, The First Affiliated Hospital of Harbin Medical University, Harbin 150000, China

Y. Sun · T. Sun · J. Zhang · X. Chen · Q. Li · S. Sun
Department of Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin 150000, China

M. Liang (✉)
Department of Neurobiology, Harbin Medical University Provincial Key Lab of Neurobiology, Harbin Medical University, Harbin 150086, China
e-mail: lm1978081@126.com

M. Liang
e-mail: lm19780816@126.com

Introduction

Colorectal cancer, caused by a variety of factors, is extremely harmful to human health, but its mechanism is still not well defined [1]. Immunodeficiency, lack of host response to tumor antigens, contributes to the evasion of colorectal cancer cells from host antitumoral immunity [2–5]. Thus, there has been significant interest in tumoral immunology research [2–5]. T-cell-mediated immunity plays an important role in the anti-colorectal cancer immunity [2–5]. T cell activation requires TCR-mediated antigen signal and co-stimulatory molecules together to provide the second signal [2–6].

B7 family is an important costimulatory molecule [4–6]. B7-H1 and B7-H4 are newly discovered B7-CD28 family of negative co-stimulatory molecules [4–6]. They can inhibit T cell activation and proliferation to negative regulation of T cell immune response [5–11]. Although expression of B7-H molecules is principally limited to lymphoid cells, aberrant expression by a number of human malignancies has been shown [5–13]. Additionally, there is increasing evidence that suggests that these B7-H molecules may serve as co-inhibitors of cell-mediated immunity. As such, B7-H expression has been correlated with aggressive behavior by various tumors.

B7-H1, also known as programmed death ligand-1 (PD-L1), can be induced in most peripheral hematopoietic tissues [14–18]. Engagement of B7-H1 with its receptor PD-1 on T cells delivers a signal that inhibits T and B cell proliferation [14–18]. PD-1 is a member of the immunoglobulin superfamily,

and widely expressed in various lymphoid tissue and non-lymphoid tissues [14–18]. Recently, B7-H1 and its receptor have been shown to be highly expressed on many tumor cell surfaces, which lead to the inhibitory of cellular and tumoral immunity [14–18]. It appears that up-regulation of B7-H1 is a mechanism that cancers can employ to evade the host immune system by inducing the death of CTL [14–18]. Recent studies have showed than the blockage of B7-H1 and PD-1 signaling pathway can effectively inhibit tumor growth, which may be an effective immunal treatment for tumor [14–18].

B7-H4 is a ligand within the B7 family that has been implicated as a negative regulator of T-cell-mediated immunity [19–26]. Weak or sporadic expression of B7-H4 has also been observed in some peripheral tissues. BTLA expression is induced during activation of T cells which may increase the expression of B7-H4 receptor [15–24]. B7-H4 acts as a negative regulator of T cell responses by inhibiting T cell proliferation, cell cycle progression, and cytokine production [18–26]. B7-H4 negative regulates cellular immune response [6, 10–25]. Human cancers of the lung, breast, and ovary have also been shown to aberrantly over-express the B7-H4 protein ligand [5–22].

Collectively, these findings suggest that B7-H1 and B7-H4 may function as a negative regulator of immune responses through different pathways such as regulating T cell proliferation, cell cycle progression, cytokine production, and induction of tumor cell apoptosis. However, studies pertaining to colorectal tumor expressions of B7-H1 and B7-H4 have not been performed. In this report, we elucidate possible mechanisms whereby B7-H1 and B7-H4 react with T cells and tumor-associated macrophages (TAM) which causing tumors evade host antitumoral immunity. These results may suggest the blockage of these signal pathways by genetic engineering or medicine as a treatment for colorectal cancer. In the present study, immunohistochemistry was performed to measure the expressions of B7-H1 and B7-H4 in colorectal cancer. The expressions of these markers were correlated with clinicopathological factors and prognosis of colorectal cancer.

Materials and methods

Study population

Informed consent was obtained before the study; all samples from patients and normal subjects (in northeast China's Heilongjiang province) were collected according to the procedures approved by the institutional review board of Second Affiliated Hospital of Harbin Medical University, China. Forty-four patients with colorectal cancer who underwent surgical treatment at the Department of Surgery were enrolled in this study. Preoperative clinical and laboratory features included age, sex, infiltrating depth, primary tumor classification, regional lymph node involvement as well as distant metastasis. Samples were stored in liquid nitrogen, −80 °C. The classification of invasive cancer was performed according to the Scarff–Bloom–Richardson system. On the basis of frequency of cell mitosis, tubule formation, and nuclear pleomorphism, invasive cancers can be graded into low grade (grade I), moderate grade (grade II), and high grade (grade III). Lymph node metastasis was also recorded in each patient. According to the AJCC in Handbook of Cancer Stages (6th edition), the TNM stage was also determined. The median age of these patients was 52 years (range, 29–72 years).

Immunohistochemistry and staining evaluation

Both the percentage of positive tumor cells and the intensity of staining were assessed in a semiquantitative fashion, and the slide was assigned a total score based on both results. Polyclonal antibodies against B7-H1 and B7-H4 were prepared by immunization of rabbits against synthetic peptides in cooperation with Santa Cruz Biotechnology. In all samples, the technique was performed following standard procedures: formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene, hydrated through graded alcohol, and rinsed in phosphate-buffered saline (PBS). Paraffin-embedded specimens were treated with sodium citrate buffer pH: 6.0 at 100 °C for 10 min and incubated overnight at 4 °C in primary anti-B7-H1 and B7-H4 antibody with dilution of 1:100. Specimens were next washed with PBS. For the subsequent reaction, the tissue sections were treated with biotinylated secondary antibody for 30 min, followed by further incubation with streptavidin–horseradish peroxidase complex. Signals were visualized with DAB and H_2O_2 for 10 min and then the slides were counterstained with hematoxylin.

Semiquantitative expression levels were based on staining intensity and distribution [13]. In cytoplasm, staining intensity was graded as 0 (no staining), 1 (weak staining=light yellow), 2 (moderate staining=yellow brown), and 3 (strong staining=brown). The percentage (0–100 %) of the extent of reactivity scored as follow: 0 (no positive tumor cells); 1 (less than 10 % positive tumor cells); 2 (10–50 % positive tumor cells); and 3 (more than 50 % positive tumor cells). Next, the cytoplasmic expression score was obtained by multiplying the intensity and reactivity extension values. Patients were classified into two groups based on measuring heterogeneity by the log-rank test with regard to overall survival. The scores exhibiting less than 4 were classified as low expression and the remainder as high expression.

Follow-up

Clinical and pathological factors were evaluated, and all patients were followed for 5 years. Among 185 patients with colorectal cancer, two were lost to follow-up due to change

of residence, and the remaining 183 patients were followed for at least 5 years or until they died. In the present study, disease-free survival rate (DFS) and overall survival rate (OS) were analyzed.

Statistical methods

Statistical analyses were done using the SPSS software package (SPSS Institute). Associations of B7-H1 and B7-H4 expressions with clinical and pathologic features were evaluated using Spearman rank correlation analysis and χ^2 analysis. Survival curves were plotted by the Kaplan–Meier method, and differences were assessed by the log-rank test. Univariate analysis was performed by means of the log-rank test to determine the differences between levels of potential prognostic variables. To assess independent impact of gene expression on DFS and OS, multivariate analysis was also performed using the Cox proportional hazard regression model applied in a stepwise forward mode. Significance values of $p<0.05$ were considered statistically significant. Results are expressed as the mean ± standard deviation.

Results

B7-H1 and B7-H4 expressions in colorectal tissues

B7-H1 and B7-H4 were expressed in cytoplasma and membrane of the tumor cells, especially in the intestinal gland epithelial cells, but tumor-infiltrating lymphocytes were devoid of staining (Fig. 1). In 39 normal colorectal tissues adjacent to the tumor samples, there were four (10.26 %) tumors that were B7-H1-positive, and three (7.70 %) that were B7-H4-positive.

There was significant difference between normal colorectal tissues and tumor tissues.

Association of B7-H1 and B7-H4 expressions as well as tumoral T cell infiltration

CD3- and CD8-positive tumor infiltrating T lymphocytes in colorectal cancer tissues were located in the membrane. Most CD3$^+$ T, CD8$^+$ T cells gathered in the surrounding tissues adjacent to the tumor, a small number infiltrated the tumor and directly contact with tumor cells. The number of CD3$^+$ T cells (83.3±15.7) in B7-H1-positive specimens were significantly lower than that (100.8±14.3) in B7-H1-negative specimens ($p<0.01$). But there were no significant difference between CD8$^+$ T cells (43.1±9.7) in B7-H1-positive specimens and CD8$^+$ T cells (51.3±12.7 B7-H1) negative specimens ($p=0.071$).

The number of CD3$^+$ T cells and CD8$^+$ T cells (81.5±12.9 and 41.4±8.5) in B7-H4-positive specimens were significantly lower than that (110.9±11.3 and 59.8±6.5) in B7-H4-negative specimens.

Association of combined tumor B7-H1 and B7-H4 expression with tumor-associated macrophages

CD68-positive macrophages were found in the cytoplasm and membrane of the tumor. CD68$^+$ TAM distributed in the tumor stroma and some infiltrated into tumor substance. Macrophages in mesenchymal did not express B7-H4, weak expression of B7-H4 was found in tubular macrophages. CD68$^+$ TAM in B7-H1-positive specimens (58.1±17.0) had no significant difference with that in B7-H1-negative specimens (46.7±23.4, $p=0.119$). CD68$^+$ TAM in B7-H4-positive specimens (60.0±15.2) were significantly higher than that in B7-H4-negative specimens (37.1±22.5, $p<0.01$).

Fig. 1 B7-H1 and B7-H4 expressions in colorectal tissues. **a** B7-H1 positive in colorectal cancer specimens (×200). **b** B7-H4 positive in colorectal cancer specimens (×200). **c** Both B7-H1 and B7-H4 negative in colorectal cancer specimens (×400). **d** Both B7-H1 and B7-H4 negative in normal colorectal specimens (×400)
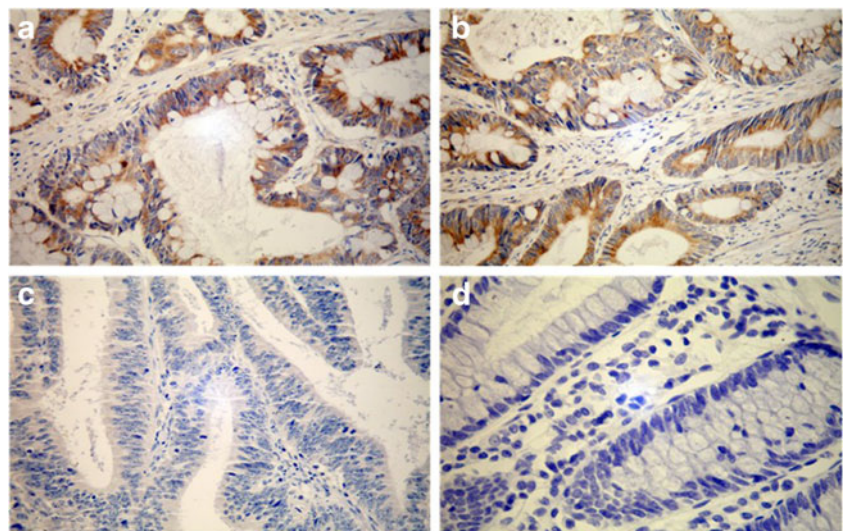
Clinical and pathologic features by tumor B7-H1 and B7-H4 expressions

Clinical and pathologic features of 185 colorectal cancer patients were observed. Positive tumor B7-H1 and B7-H4 expression was associated with lymph node metastasis ($p<0.05$), but have no direct relation with age, gender, infiltrating depth, and tumor stage and grade ($p>0.05$, Table 1).

Univariate and multivariate analysis of the prognosis of Heilongjiang patients with colorectal cancer

In the present study, univariate and multivariate survival analysis was performed to evaluate the influence of the expression of B7-H1 and B7-H4, and clinicopathological factors (age, clinical grade, histological grade, lymph node metastasis, and TNM stage) on the prognosis of patients with colorectal cancer. The analysis showed that the poor prognosis of colorectal cancer was unrelated to age, histological

grade, or tumor size; however, the disease-free survival rate and overall survival rate were associated with the lymph node metastasis, TNM stage, and expressions of B7-H1 and B7-H4 (Table 2).

Effect of B7-H1 and B7-H4 on patients' survival

The Kaplan–Meier 5-year survival curves stratified for B7-H1 and B7-H4 expressions are shown as follows. Patients whose tumors had high expression of B7-H1 and B7-H4 had a significantly lower 5-year disease-free survival rate when compared with those whose tumors had B7-H1 and B7-H4 expressions (Log-rank; $p<0.01$). Moreover, patients whose tumors had high B7-H1 and B7-H4 expressions had markedly shorter overall survival time when compared with patients whose tumors had low B7-H1 and B7-H4 expressions (Log-rank; $p<0.01$).

Discussion

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of death around the world [1]. Immune system deficiencies, lack of host response to tumor antigens as well as tumor cells evade from the immunal supervision are the main factors in the occurrence of colorectal cancer [3–15]. Mechanism of tumoral immunal evasion is an area of active research interest [2–9].

B7-H1 and B7-H4 are newly discovered B7 family members providing negative signals to limit, terminate, or reduce T-cell immune response [6–9]. Engagement of B7-H1 with its receptor PD-1 on T cells delivers a signal that inhibits TCR-mediated activation of IL-2 production and T cell proliferation and plays an important role in the tumoral immunal evasion [14–17]. Although B7-H4 mRNA has been noted in a number of nonlymphoid organs such as kidney, uterus, testis, liver, and spleen, but weak or sporadic expression of B7-H4 protein has been observed in somatic tissues [6–25]. Additionally, B7-H4 has been found to be aberrantly expressed at high levels in human tumor cells [6–25]. This over-expression of B7-H4 by malignant tissues may render B7-H4 a particularly useful target for facilitating antitumoral immunotherapeutic responses [4–25].

Our current study demonstrated that cytoplasma and membrane of the tumor cells, especially the intestinal gland epithelial cells, expressed B7-H1 and B7-H4, but tumor-infiltrating lymphocytes were devoid of staining. The B7-H1- and B7-H4-positive rates were significantly higher than that in the normal colorectal tissue adjacent to the tumor. Paralleling results were obtained by other investigators. The combined expressions of B7-H1 and B7-H4 within the same tumor cells were associated with the occurrence of colorectal cancer [3–25]. Krambeck et al. [18] have reported that the

Table 1 Clinicopathological features associated with B7-H1 and B7-H4 expressions in 185 Heilongjiang patients with colorectal cancer

	B7-H1		p value[a]	B7-H4		[a]p value
	Over	Low		Over	Low	
Patient no. (185)						
Gender						
Male	52	35	0.2324	57	33	0.1580
Female	50	48		60	35	
Age (years)						
≥60	62	52	0.7952	66	32	0.8914
≤59	40	31		51	36	
Histology grade						
I	73	52	0.1975	33	36	0.2167
II/III	29	31		84	32	
Stage						
I/II	27	45	0.0001	23	43	<.0001
III/IV	75	38		94	25	
B7-H4						
High	84	33	<.0001			
Low	18	50				
Tumor size						
T1	23	43	<.0001	21	40	0.0001
T2/T3	79	40		96	28	
Lymph node status						
N0/N1	26	51	<.0001	22	57	<.0001
N2	76	32		95	11	
LVS invasion						
Yes	64	13	<.0001	89	8	<.0001
No	38	70		38	60	

LVS lymphatic vessel size

[a] p values by Pearson's chi-square test

Table 2 Univariate and multivariate analysis of the prognosis of Heilongjiang patients with colorectal cancer

Variables	DFS		OS	
	HR (95 %CI)	p	HR (95 %CI)	p
Age (years) <50/≥50	1.634 (0.975–2.473)	0.0692	1.512 (0.968–2.361)	0.0714
Gender male/female	1.350 (0.681–2.702)	0.5014	1.264 (0.635–2.514)	0.6153
LVS invasion no/yes	1.810 (1.101–2.610)	0.0172	1.670 (1.018–2.514)	0.0571
Tumor size >2 cm/≤2 cm	1.168 (0.480–2.837)	0.7325	1.153 (0.391–2.726)	0.7590
Histology grade I/II III	1.714 (0.934–2.913)	0.0845	1.605 (0.920–2.802)	0.0917
Lymph node N0 N1/N2	1.732 (1.108–2.706)	0.0159	1.691 (1.005–2.691)	0.0406
TNM stage I/II III	1.847 (1.219–2.841)	0.0095	1.751 (1.207–2.746)	0.0180
B7-H1 expression Low/ high	1.831 (1.214–2.806)	0.0049	1.740 (1.195–2.713)	0.0142
B7-H4 expression low/high	1.829 (1.195–2.819)	0.0057	1.729 (1.207–2.663)	0.0150

LVS lymphatic vessel size

combined expressions of B7-H1 and B7-H4 associated with an increased risk of death in patients with renal tumors [14–24]. As a corollary to this, we surmise that assessment of combined B7-H1 and B7-H4 expression within colorectal tumors may be useful in discriminating between patients who are most likely to benefit from immunotherapy versus alternate forms of systemic therapy [14–24].

Tumor-infiltrating lymphocytes (TILs) are responsible for tumor killing and may induce spontaneous regression [4–24]. Cytotoxic T cell (also known as CTL, CD8^{+} T-cells, or killer T cell) are capable of inducing the death of infected somatic or tumor cells [5–10]. The presence of TIL correlates with a better prognosis in patients with several types of cancer, and CD3^{+}, CD8^{+} T lymphocyte subset has a unique role in the antitumor response [5–10]. Kondratiev et al. [19] indicated that the infiltrating CD8^{+} T lymphocytes in patients with endometrial cancer are an independent prognostic factor for survival, as well in colorectal cancer [19–24].

In vitro, negative co-stimulatory molecule B7-H1 and B7-H4 have the ability to inhibit CD4^{+} and CD8^{+} T cell proliferation and activation, induce apoptosis, and reduce the secretion of IL-2, 10, and IFN-γ [5–19]. Up to now, immunohistochemical method has been used to identify the relationship between the presence of TILs and the expressions of B7-H1 and B7-H4. B7-H1 expression has been correlated with non-small cell lung cancer and ovarian carcinoma [12–25]. Miyatake et al. [9] indicated that the expression of B7-H4 in endometrial carcinoma correlated with the numbers of CD3^{+} T and CD8^{+} T cells.

In this report, we test the number of TILs [19–23] to elucidate the effect of the expressions of B7-H1 and B7-H4. In B7-H1, B7-H4-positive colorectal cancer samples, the number of CD3^{+} T cells and CD8^{+} T cells was significantly lower than the number of negative group. CD3^{+} T cells number was negatively correlate with B7-H1^{+} expression. CD3^{+} T, CD8^{+} T cells number was negatively correlated with B7-H4^{+} expression. These results suggest that B7-H1 and

B7-H4 is a mechanism that cancers can employ to evade the host immune system by regulating the differentiation of T cells.

Tumor-associated macrophages can promote tumor cell proliferation, inhibit activity of natural killer cells, and T lymphocytes and play a important role in the prognosis and progress of tumor [19–26]. Kryczek [25] found that B7-H4 express on the surface of TAM in ovarian cancer tissues. As a contrast, in our experiments, we only found sporadic B7-H4 expression in cytoplasm but not on membrane. These findings suggest that macrophages absorb B7-H4 by the phagocytic mechanism instead of expressing it. So, further experiments are needed to confirm the source of B7-H4 expression. The relationship between the B7-H1, B7-H4 expression, and TAM number and the reacting mechanism remain unclear. Our studies showed that CD68^{+} TAMs in B7-H1^{+}, B7-H4^{+} samples are significantly higher than the number of negative group, which suggest that enhanced inflammatory response in B7-H1[+] and B7-H4[+] samples lead to more TAMs which induce antitumoral immunity. TAM has also expressed the lymphatic endothelial growth factor receptor (VEGF-C), which is related to peritumoral lymphangiogenesis [22–26].

In the present study, we found that the expression of B7-H1 and B7-H4 were related to the prognosis of colorectal cancer. Patients whose tumors had high expression of B7-H1 and B7-H4 had poorer DFS and OS when compared with those whose tumors had low expression of B7-H1 and B7-H4. Furthermore, patients whose tumors had high B7-H1 and B7-H4 expression had significantly poorer prognosis than did those whose tumors had low B7-H1 and B7-H4 expressions. At the same time, patients whose tumors had low B7-H1 and B7-H4 expressions had a prognosis superior to that of those whose tumors had high B7-H1 and B7-H4 expressions.

In summary, these findings suggest that B7-H1 and B7-H4 may function as a negative regulator of immune responses through different pathways, which causing tumors

evade host antitumoral immunity. We surmise that interference with the mediated T cell co-stimulatory signal transduction pathway by blocking B7-H1 and/or B7-H4 pathways may lead to future immunal therapeutics for the treatment of tumor. Our study has also demonstrated that increased expressions of the B7-H1 and B7-H4 protein can result in increased LVS invasion and metastasis, which was closely related to the poor prognosis of patients with colorectal cancer; however, our study had some limitations. We recruited colorectal cancer patients only in our hospital (Heilongjiang province). A multi-center study would be preferable. In addition, only histological examination was performed in our study, and in vivo study was not performed.

Conflicts of interest None

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