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Clinical Significance of the B7-H4 Coregulatory Molecule as a Novel Prognostic Marker in Gastric Cancer

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Abstract
Background The B7-H4 coregulatory molecule is a member of the B7 family of molecules, which regulate the T-cell-mediated immune response through CD28 receptors. Recently, B7-H4 has been reported to be a negative regulator of the immune response in patients with several malignant diseases. However, few reports have investigated the clinical significance of B7-H4 expression in patients with gastric cancer. In the present study, we analyzed B7-H4 expression and the relationship between its expression and clinicopathological factors including prognosis in gastric cancer.
Methods B7-H4 expression in gastric cancer cell lines and clinical gastric cancer specimens was initially assessed with the reverse transcription-polymerase chain reaction (RT-PCR). Moreover, B7-H4 and CD3 expression in 120 resected specimens from gastric cancer patients were evaluated by immunohistochemistry (IHC).
Results B7-H4 expression was identified in the gastric cancer cell lines and clinical tumor tissues by RT-PCR. B7-H4 expression was high in 25.8% (31/120) of resected tumor specimens. B7-H4 expression significantly correlated with tumor stage ($P = 0.04$). The 5-year survival rate was significantly lower in patients with high B7-H4 expression than in those with low B7-H4 expression ($P = 0.001$). Multivariate analysis demonstrated that B7-H4 expression was an independent prognostic factor ($P = 0.035$). Immunohistochemical analysis of CD3 expression showed that B7-H4 expression was inversely correlated with the number of tumor infiltrating T lymphocytes ($P < 0.001$).
Conclusions The B7-H4 coregulatory molecule is a novel prognostic marker related to the T-cell-mediated immune response, and its pathway may be a molecular target for controlling tumor progression in patients with gastric cancer.

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Introduction

Gastric cancer is the leading cause of cancer-related deaths and the most common gastrointestinal tract carcinoma in Japan [1–3]. Recently, a randomized controlled trial indicated that adjuvant S-1 chemotherapy following curative surgery (R0) is a useful treatment for patients with stage II or III gastric cancer [4]. However, it is difficult to predict postoperative occult recurrence with standard blood and imaging examinations such as ultrasonography, computed tomography, and positron emission tomography [5]. Furthermore, there are no biomarkers for predicting the prognosis or therapeutic response of patients with advanced gastric cancer. Accordingly, molecular markers of disease recurrence are required for the clinical management of postoperative patients with gastric cancer.

In recent years, members of the B7 family have been reported to control the T-cell-mediated immune response [6–8]. The regulatory signals generated by the interaction between these B7 family members and their CD28 receptors on activated T cells have a powerful impact on the immune surveillance system [6–8]. To date, several B7 family members have been identified, and the B7-H4 coregulatory molecule is a newly identified [9–11]. Several

investigators have demonstrated that B7-H4 plays an important role as a negative modulator of the immune response in patients with malignant disease [12–21]. Furthermore, B7-H4 has been reported to be a molecular biomarker associated with tumor progression and survival in patients with ovarian cancer and renal cell cancer [14, 16, 17]. Additionally, B7-H4 expression has been confirmed in breast cancer, pancreatic cancer, and non-small-cell lung cancer tumors [12, 15, 21]. We previously investigated B7-H4 mRNA expression in the peripheral blood of gastric cancer patients and demonstrated that B7-H4 expression in blood specimens is significantly correlated with tumor progression, including prognosis in gastric cancer patients [22]. However, few reports have revealed the relationship between B7-H4 expression in primary tumors and tumor properties in gastric cancer patients.

The purpose of the present study was to assess B7-H4 expression in primary gastric tumors and to investigate the relationship between B7-H4 expression and clinicopathological findings, including prognosis in gastric cancer patients.

Materials and methods

Gastric cancer cell lines

The gastric cancer cell lines, MKN-7, MKN-45, MKN-74, KATO-III, and NUGC-4, were cultured in RPMI 1640 (Roswell Park Memorial Institute medium; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Mitsubishi Kasei, Tokyo, Japan), 100 units/ml penicillin, and 100 units/ml streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2, as described elsewhere [23, 24]. These cell lines were used for the reverse transcription-polymerase chain reaction (RT-PCR) assay.

Patients and specimens

We enrolled 120 patients (74 men and 46 women; age range: 31–83 years; mean: 65 years) with gastric cancer who underwent curative gastrectomy combined with lymphadenectomy at Kagoshima University Hospital (Kagoshima, Japan) between 2000 and 2005. Patients who had undergone endoscopic mucosal resection, palliative resection, preoperative chemotherapy, and/or radiation therapy were excluded from this study. Furthermore, none of the patients enrolled in this study had synchronous or metachronous cancer in other organs. The tumors were classified and staged based on the criteria for the tumor-node-metastasis (TNM) classification of gastric carcinoma established by the International Union Against Cancer (UICC) [25]. After being discharged, all patients were followed-up every 3–6 months with tumor marker studies (CEA and CA19-9), radiography, ultrasonography, and computed tomography at Kagoshima University Hospital. The median follow-up period after surgery was 40 months (range: 1–112 months).

Resected primary tumors were fixed with 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned into 3 μm slices. Paraffin-embedded archival tissue (PEAT) specimens obtained from these resected primary tumors were histopathologically confirmed by a surgical pathologist. In these 120 resected primary tumors, three fresh gastric cancer specimens were used for the RT-PCR assay.

All specimens were collected from patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital.

RNA extraction and RT-PCR assay

Fresh tumor specimens were homogenized in FastPrep (Qbiogene, Inc., Carlsbad, CA, USA). Total RNA was extracted, isolated, and purified in phenol-chloroform as described elsewhere [23, 24]. The concentration and purity of the total RNA were determined using a GeneQuant pro UV/Vis spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). The primer sequences of B7-H4 and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were designed for RT-PCR assays of each marker. The primers for B7-H4 and GAPDH were as follows: B7-H4: 5′-CTTCTGCCTCTCAGCCCTTA-3′ and 5′-GAAATAGT TCTGTAGATCCCTGTTG-3’, and GAPDH: 5’-GGGTG TGAACCATGAGAAGT-3′ and 5′-GACTGTGGTCATG AGTCCT-3′. The integrity of the RNA was certified by RT-PCR assay using GAPDH.

All total RNA samples were reverse transcribed using the Advantage RT-for-PCR kit (Clontech Laboratories, Inc., Palo Alto, CA), as described elsewhere [23, 24]. The RT-PCR assay was performed with the GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA). The amplification profile comprised precycling at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s (56°C for B7-H4 and 55°C for GAPDH), and extension at 72°C for 30 s before a final extension step at 72°C for 10 min. The RT-PCR products were verified with a 2% agarose gel. Each assay was repeated in triplicate with a negative (H_2O) control.

Immunohistochemical staining

Paraffin embedded archival specimen sections (3 μm thick) of the resected primary tumors were incubated on slides at

50°C overnight, deparaffinized with xylene, and then rehydrated with a graded series of ethanol. After being washed in PBS, the sections were autoclaved in ethylenediaminetetraacetic acid (EDTA) buffer (1 mM, pH 8.0) at 120°C for 10 min to activate the antigen and immersed in peroxidase-blocking solution (DAKO Corporation, Carpinteria, CA) for 10 min to block endogenous peroxidase, washed three times for 5 min each with PBS, and then nonspecific binding was blocked with protein-blocking serum-free solution (DAKO) at room temperature for 30 min. The sections were then incubated at 4°C overnight with anti-human B7-H4 antibody (Abbiotec, LLC, San Diego, CA) diluted 1:500 in PBS. After being incubated with PBS three times for 5 min each, the reactions for B7-H4 were developed by the ABC method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) [26] and visualized with diaminobenzidine tetrahydrochloride (DAB). Negative controls were treated with PBS without primary antibody under the same conditions.

In the immunohistochemical procedure for CD3, the antigen was activated by DakoCytomation Proteinase K (DAKO) at room temperature for 10 min, and the sections were immersed in peroxidase-blocking solution (DAKO) for 10 min to block endogenous peroxidase activity; nonspecific binding was blocked with protein-blocking serum-free solution (DAKO) at room temperature for 30 min. The sections were incubated at room temperature for 60 min with anti-human CD3 antibody (DAKO) diluted 1:100 in PBS. The CD3 reactions were developed with the ABC method and visualized with DAB [26].

Evaluation of immunohistochemical findings

Two independent investigators (T.A. and Y.U.) who were blinded to the patients' clinicopathological data performed the immunohistochemical analysis. Based on the appraisal criteria for immunostaining intensity established by a previously published article, B7-H4 immunoreactivity was classified into four groups: negative immunoreaction (−), weak immunoreaction (+), moderate immunoreaction (++), and strong immunoreaction (+++) [27].

On the other hand, an analysis of CD3 immunohistochemical staining was performed to assess the number of tumor infiltrating T lymphocytes in primary tumor foci. These tumor infiltrating T lymphocytes were counted in five fields using light microscopy (magnification, ×200). The number of tumor infiltrating T lymphocytes recorded in five fields were averaged and used in the statistical analysis.

Statistical analysis

The Kendall *W* coefficient of concordance was used to assess an agreement of immunohistochemical findings by two investigators. To compare categorical clinicopathological factors, group differences based on B7-H4 expression status were statistically analyzed with the chi-square and Fisher's exact tests. Differences in tumor infiltrating T lymphocytes between the high and low B7-H4 expression groups were assessed with the Wilcoxon rank sum test. Survival curves were generated with the Kaplan-Meier method, and differences in survival were examined with the log-rank test. Prognostic factors were assessed by univariate and multivariate analyses (Cox proportional hazard regression model). All statistical calculations were performed with SAS statistical software (SAS Institute. Inc., Cary, NC). A P value of <0.05 was considered statistically significant.

Results

B7-H4 expression in cell lines and clinical tumor tissues based on RT-PCR

Initially, B7-H4 expression in five gastric cancer cell lines and in three tumor specimens from gastric cancer patients was assessed with the RT-PCR assay.

Although the MKN-45 cell line displayed weak B7-H4 expression, B7-H4 mRNA expression was identified in all cell lines (Fig. 1). In clinical gastric tissues, B7-H4 mRNA

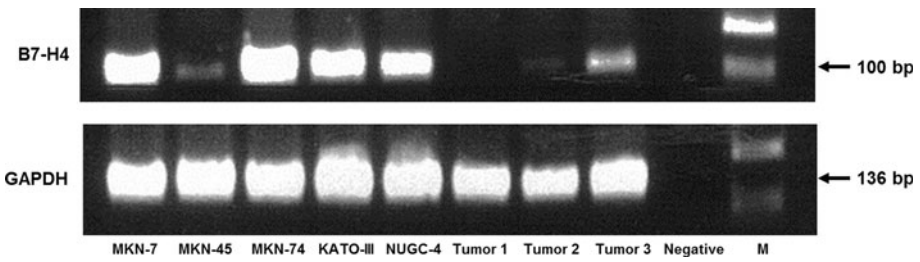

Fig. 1 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of B7-H4 mRNA expression in gastric cancer cell lines and clinical tumor tissues. B7-H4 mRNA expression (100 bp) was identified in all cell lines and one clinical tumor specimens. *M* DNA molecular weight marker

expression was confirmed in one of three tumor specimens (Fig. 1).

B7-H4 expression in primary gastric tumors based on immunohistochemistry

The B7-H4 expression in 120 PEAT specimens obtained from patients with gastric cancer was assessed by immunohistochemical staining. Interobserver agreement in the assessment of immunohistochemical findings was excellent ($W = 0.85$, $P < 0.01$).

B7-H4 expression was detected in the membrane and/or cytoplasm of the tumor cells. B7-H4 was defined as negative immunoreaction in 7 cases, weak immunoreaction in 82 cases, moderate immunoreaction in 25 cases, and strong immunoreaction in 6 cases (Fig. 2). In the present study, high expression was defined as the presence of moderate or strong B7-H4 immunoreactivity. Therefore, high B7-H4 expression was identified in 31 (25.8%) of 120 PEAT specimens.

B7-H4 expression and clinicopathological factors

To evaluate the relationship between B7-H4 expression and clinicopathological findings, all patients were classified into one of two groups based on their B7-H4 immunoreactivity (high group, $n = 31$; low group, $n = 89$). B7-H4 expression was significantly correlated with UICC stage ($P = 0.04$; Table 1), but not with any other clinicopathological factor.

B7-H4 expression and prognosis

The 5-year survival rates of patients with high and low B7-H4 expression were 34.7% and 80.4%, respectively (Fig. 3). The 5-year survival rates were significantly lower in patients with high B7-H4 expression than in those with low B7-H4 expression ($P = 0.001$).

Univariate analysis demonstrated that histological type, depth of tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion, and B7-H4 expression were significantly related to postoperative survival ($P = 0.01$, <0.01, <0.01, <0.01, <0.01 and <0.01, respectively; Table 2). However, only B7-H4 expression was found to be an independent prognostic factor in multivariate analysis ($P = 0.035$; Table 2).

B7-H4 expression and tumor infiltrating T lymphocytes

To investigate the relationship between B7-H4 expression and tumor immune surveillance, the number of tumor infiltrating T lymphocytes was assessed by CD3 immunohistochemical staining.

Tumor infiltrating T lymphocytes stained by CD3 antigen were diffusely identified in tumor foci (Fig. 4). The mean number of tumor infiltrating T lymphocytes (± SD) was 55.7 ± 36.8 in tumors with high B7-H4 expression and 98.6 ± 62.4 in tumors with low B7-H4 expression (Fig. 5). Consequently, the B7-H4 expression status of primary tumor cells was inversely correlated with the number of tumor infiltrating T lymphocytes ($P < 0.001$).

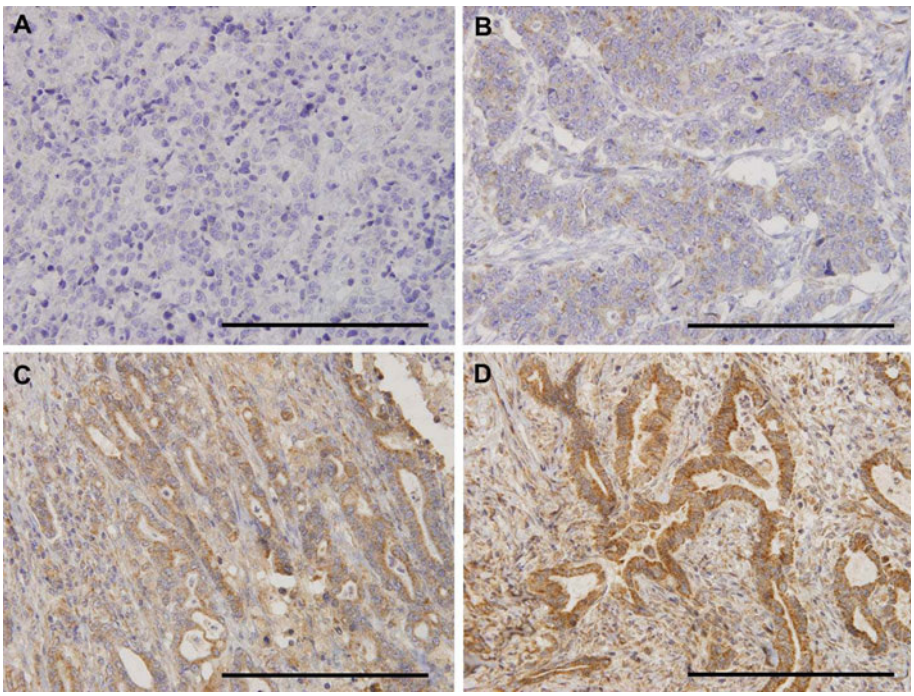

Fig. 2 Representative immunohistochemical staining of B7-H4 expression in gastric tumor tissue. Tumor cells with negative (**a**), weak (**b**), moderate (**c**), and strong (**d**) expression of B7-H4. Scale bars indicate 200 μm. Original magnification ×400

Table 1 Relationship between B7-H4 expression and clinicopathological features in gastric cancer patients

Variable	B7-H4 expression (%)		
	Low ($n = 89$)	High ($n = 31$)	P value
Gender			
Male	55 (61.8)	19 (61.3)	1.000
Female	34 (38.2)	12 (38.7)	
Age (years)			
≤70	49 (55.1)	23 (74.2)	0.088
>70	40 (44.9)	8 (25.8)	
Histological type			
Differentiated	38 (42.7)	12 (38.7)	0.833
Undifferentiated	51 (57.3)	19 (61.3)	
Depth of tumor invasion			
pT1–T2	41 (46.1)	8 (25.8)	0.058
pT3–T4	48 (53.9)	23 (74.2)	
Lymph node metastasis			
Negative	45 (50.6)	9 (29.0)	0.058
Positive	44 (49.4)	22 (71.0)	
Stage			
I–II	51 (57.3)	11 (35.5)	0.040
III–IV	38 (42.7)	20 (64.5)	
Lymphatic invasion			
Negative	33 (37.1)	7 (22.6)	0.185
Positive	56 (62.9)	24 (77.4)	
Venous invasion			
Negative	45 (50.6)	13 (41.9)	0.532
Positive	44 (49.4)	18 (58.1)	

pT1 invasion of lamina propria or submucosa, *pT2* invasion of muscularis propria, *pT3* invasion of subserosa, *pT4* penetration of serosa without invasion of adjacent structures or invasion of adjacent structures

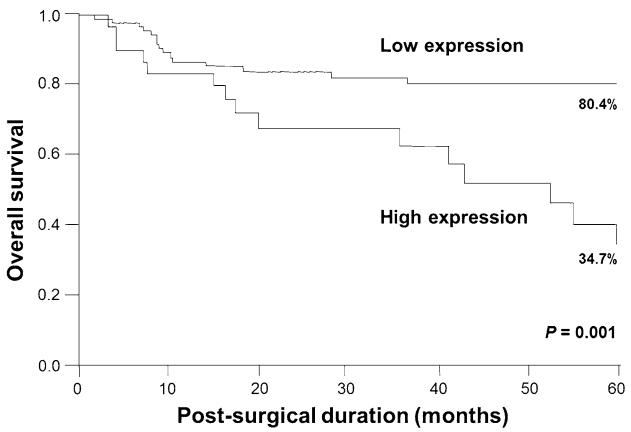

Fig. 3 Kaplan-Meier survival curves for gastric cancer patients based on B7-H4 expression. Patients with high B7-H4 expression had a significantly poorer prognosis than those with low B7-H4 expression ($P = 0.001$)

Discussion

In the present study, using immunohistochemical staining, we demonstrated B7-H4 protein expression in primary tumor cells from patients with gastric cancer. Furthermore, the relationship between B7-H4 expression and tumor properties was assessed to investigate the role of B7-H4 ligand in the immune response of gastric cancer patients. To date, few studies have focused on the clinical impact of B7-H4 expression in gastric cancer.

The B7-H4 molecule was recently identified and characterized as a novel B7 family member [9–11]. B7-H4 mRNA is widely expressed in peripheral tissues, such as the placenta, liver, skeletal muscle, kidney, pancreas, prostate, testis, ovary, and small intestine [9, 28]. On the other hand, B7-H4 protein expression is limited in normal human tissues [9, 28]. Previous immunohistochemical reports have demonstrated that B7-H4 protein is expressed in 95.4 and 42.9% of patients with breast and lung cancer, respectively [12, 15]. Recently, the B7-H4 molecule has been focused on as a candidate serum and tissue biomarker in ovarian cancer [13, 14, 17, 20]. Furthermore, the measurement of B7-H4 expression is expected to become a useful tool for the prediction of chemotherapeutic response and prognosis in ovarian cancer patients receiving chemotherapy [29]. In pancreatic cancer, it has been reported that B7-H4 has potential utility as a diagnostic marker for detecting tumor cells in specimens obtained from endoscopic ultrasound-guided fine-needle aspiration [21]. In the present study, primary gastric tumor cells displayed various degrees of B7-H4 immunoreactivity, and its expression was observed in 113 (94.2%) of 120 patients with gastric cancer. These results indicate that the majority of patients with gastric cancer express B7-H4 and that it may be a useful diagnostic marker for patients with gastric cancer.

In this study, although no significant relationship was detected in the statistical analysis, patients with high B7-H4 expression tended to display deeper tumor invasion and the presence of lymph node metastasis compared with those with low B7-H4 expression. Furthermore, B7-H4 expression in primary tumor cells was significantly correlated with UICC stage ($P = 0.04$). Our findings demonstrate a close relationship between B7-H4 expression and tumor progression in gastric cancer. Interestingly, patients with high B7-H4 expression had a poorer prognosis compared with those with low B7-H4 expression in the present study ($P = 0.001$). Moreover, B7-H4 expression was found to be a significant independent prognostic factor in multivariate analysis. Similarly, Jiang et al. reported that the status of B7-H4 expression is significantly correlated with cancer invasiveness, lymph node metastasis and prognosis in patients with gastric cancer [30]. From the viewpoint of biological tumor immune surveillance

Table 2 Univariate and multivariate analyses of survival in gastric cancer patients

Independent variable	Univariate analysis			Multivariate analysis		
	P value	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI
Histological type						
Differ/undiffer	0.01	1.86	1.20–3.21	0.221	1.34	0.85–2.35
Depth of tumor invasion						
pT1–T2/pT3–T4	<0.01	3.25	1.78–8.10	0.096	2.00	0.90–5.61
Lymph node metastasis						
Negative/positive	<0.01	2.48	1.55–4.56	0.315	1.59	0.70–6.86
Lymphatic invasion						
Negative/positive	<0.01	2.31	1.37–4.72	0.693	0.80	0.17–2.32
Venous invasion						
Negative/positive	<0.01	2.10	1.39–3.45	0.468	1.21	0.75–2.15
B7-H4 expression						
Low/high	<0.01	1.77	1.23–2.55	0.035	1.49	1.03–2.17

CI confidence interval, *differ* differentiated, *undiffer* undifferentiated

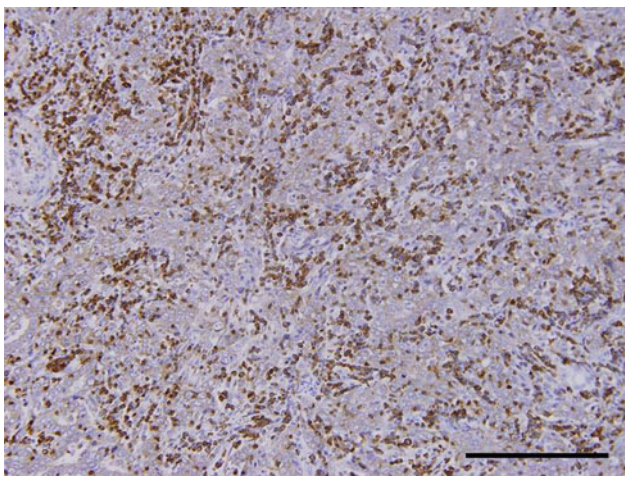

Fig. 4 Representative CD3 immunohistochemical staining for the assessment of tumor infiltrating T lymphocytes in gastric tumor foci. Tumor infiltrating T lymphocytes were diffusely identified in tumor foci. Scale bars indicate 200 μm. Original magnification ×200

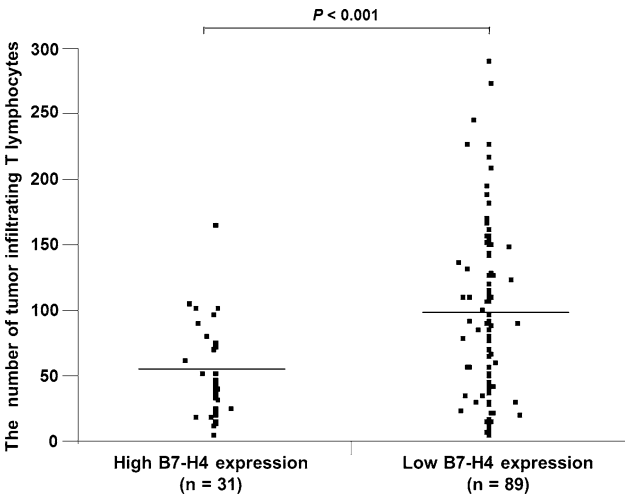

Fig. 5 Correlation between B7-H4 expression status and the number of tumor infiltrating T lymphocytes. The B7-H4 expression status was inversely correlated with the number of tumor infiltrating T lymphocytes ($P < 0.001$). Horizontal bars indicate the mean number of tumor infiltrating T lymphocytes

systems, these results suggest that the B7-H4 coregulatory molecule has the principal role as a negative regulator of these systems. Consequently, the activation of the B7-H4 signaling pathway may lead to tumor cells escaping from immune surveillance. In clinical management, our data suggest that the assessment of B7-H4 expression in primary tumor cells might yield valuable information for predicting prognosis and indicating adjuvant chemotherapy in postoperative patients with gastric cancer.

The functional role of B7-H4 expression in tumor cells remains unclear. To date, several investigators have demonstrated that the B7-H4 co-regulatory molecule inhibits T cell proliferation, cytokine secretion, and the induction of cytotoxic lymphocytes in in vitro assays [8–11]. In the present study, the number of infiltrating T lymphocytes in tumor foci was immunohistochemically assessed to investigate the mechanism behind the suppressive effect of the B7-H4 signaling pathway on the immune surveillance system. The B7-H4 expression status of primary tumor cells was inversely correlated with the number of infiltrating T lymphocytes ($P < 0.001$). Similarly, the proportion of B7-H4 positive tumor cells was inversely associated with the number of infiltrating T lymphocytes in uterine endometrioid adenocarcinoma [20]. These results strongly support the assertion that the suppressive mechanism of the

B7-H4 signaling pathway is mediated by a T-cell-mediated immune response. The identification of a receptor against the B7-H4 molecule on activated T cells would further our understanding of the B7-H4 signaling pathway and enable us to clarify the evasion system used by gastric tumors to avoid immune surveillance.

In conclusion, we demonstrated that primary gastric tumor cells express B7-H4 and that its expression is related to tumor aggressiveness, including prognosis, in patients with gastric cancer. Therefore, the B7-H4 coregulatory molecule is a potential marker for predicting malignant behavior in patients with gastric cancer. Future studies on the biological behavior of tumor cells expressing B7-H4 may lead to a new immunotherapy blocking its signaling pathway in patients with gastric cancer.

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Conflict of interest None.

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