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ORIGINAL PAPER

Peripheral blood gene expression of B7 and CD28 family members associated with tumor progression and microscopic lymphovascular invasion in colon cancer patients

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Abstract
Purpose To associate the global gene expression of B7/CD28 family transcripts with pathologic features of colon cancer, we determined the B7/CD28 family transcripts in peripheral blood mononuclear cells (PBMCs) from normal subjects and patients with adenomatous polyps and colon cancer, and correlated the results with pathologic features of colon cancer.

Methods PBMCs from age-matched normal subjects and patients with adenomatous polyps and colon cancer were analyzed for peripheral blood transcripts (PBTs) of B7/CD28 family using real-time PCR. Differences in expression levels of B7/CD28 PBTs across all cancer stages and between colon cancer patients with or without microscopic lymphovascular invasion (LVI) were analyzed.

Results The results showed a significant upregulation of PBTs of co-inhibitory molecules such as B7-H3 and PD-1 and a significant PBT downregulation of co-stimulatory molecules including CD28 and ICOS in colon cancer patients. Furthermore, the increase of B7-H3 PBT was strongly associated with tumor invasion ($P = 0.025$) and advanced TNM stages ($P = 0.019$), whereas the decline of co-stimulatory ligand B7-H2 PBT was related to regional lymph node metastasis ($P = 0.028$) and aggressive tumor invasion ($P = 0.031$). In addition, the ratios of PBT expression of CD28 family to B7 family such as CTLA-4 to B7-H2 and PD-1 to B7-H2 were significantly higher in colon cancer patients with microscopic LVI than in those without LVI ($P = 0.001$ and $P = 0.016$, respectively).

Conclusions Our results suggest that B7/CD28 family PBTs may serve as valuable markers reflecting the pathological features of colon cancer.

Keywords B7/CD28 family · Peripheral blood transcript · Tumor progression · Lymphovascular invasion

H. Lee and J. H. Kim have contributed equally.

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Introduction

Co-signaling molecules including B7/CD28 family create a dynamic network that modulates the initiation, maintenance, and termination of immune responses (Greenwald et al. 2005). Co-inhibitory ligands such as B7-H1 (PDL-1

or CD274), B7-H3 (CD276), and B7-H4 (B7x or B7S1) are ectopically expressed in non-lymphoid tissues, including various human cancers (Choi et al. 2003; Dong et al. 2002; Roth et al. 2007). These cancer-associated co-signaling molecules are believed to be involved in the immune evasion of cancer cells. B7-H1 expressed on cancer cells exerts negative effects on cytotoxic T lymphocytes (CTLs) by inducing CTL apoptosis, anergy, and functional exhaustion through an engagement of immunosuppressive PD-1 receptor on the CTLs (Dong et al. 2002; Konishi et al. 2004; Hirano et al. 2005). Recently, immunohistochemical analysis revealed that B7-H1 expressed on renal and urothelial cell carcinomas is strongly associated with cancer progression and a poor patient survival rate (Thompson et al. 2006; Boorjian et al. 2008). B7-H1 mRNA expression in cancer tissues was also reported to be of prognostic value in pancreatic cancer (Loos et al. 2008). B7-H3, a member of the B7 family, is reported to play a co-stimulatory or co-inhibitory role, as demonstrated by in vivo studies showing reduced allograft rejection in B7-H3 KO mice (Wang et al. 2005) or exacerbated autoimmune encephalitis caused by an in vivo antibody blockade of B7-H3 (Prasad et al. 2004). Although the function of B7-H3 remains controversial, the aberrant expression of B7-H3 in prostate cancer, renal cell carcinoma, and gastric carcinoma tissues is associated with poor patient prognosis (Wu et al. 2006; Zang et al. 2007; Crispen et al. 2008). Cancer-associated co-inhibitory molecules, such as B7-H1 and B7-H3, may serve as potent prognostic biomarkers for cancer patients.

Several reports have suggested that the profile of B7/CD28 family expression in peripheral blood mononuclear cells (PBMCs) is likely to reflect functional status of immune cells of patients or animal hosts with chronic viral infections; in a mouse model for lymphocytic choriomeningitis virus (LCMV) infection, PD-1 was upregulated in functionally impaired virus-specific CTLs from mice with chronic, but not acute, LCMV infection (Barber et al. 2006; Yao and Chen 2006), reflecting that PD-1 upregulation may be a hallmark of functionally exhausted CTLs in chronic viral infection. Previously, we reported that B7-H1 upregulation on monocytes in chronic hepatitis C patients is involved in suppressed T cell functions (Jeong et al. 2008), further supporting the validity of peripheral blood expression of B7/CD28 family in assessing immune status and disease progression.

Since the prognostic value of cancer-associated co-signaling molecules is based on a direct histological examination of cancer tissues, there is a need for new markers derived from PBMCs that can reflect the pathological features of cancers. In light of a significant association of B7/CD28 family expression in peripheral blood cells with disease progression in chronic viral diseases, in the present study, we analyzed the global gene expression profiles of peripheral blood transcripts (PBTs) of B7/CD28 family members that are associated with clinicopathological features such as tumor progression and microscopic invasion into lymphovascular structures.

Materials and methods

Patient selection and pathology information

All peripheral blood samples from patients and normal subjects were collected according to the procedures approved by the institutional review board of Paik Hospital, Inje University, Korea. One hundred and three patients with colon cancer who underwent surgical treatment at the Department of Surgery were enrolled in this study. Preoperative clinical and laboratory features included age, sex, and serum carcinoembryonic antigen (CEA) concentration. Pathologic features of colon cancers, including the 2002 primary tumor classification, regional lymph node involvement, distant metastasis, TNM stage groupings, and microscopic invasion into lymphovascular structure, were based on postoperative histopathologic findings (Table 1). Tumors were

Table 1 Clinical and pathologic features for colon cancer patients

Feature	*n*
Age	
>65	51
≤65	52
Sex	
Male	59
Female	44
2002 primary tumor classification	
Tis-T1	10
T2	12
T3	75
T4	5
Lymph node involvement	
N0	62
N1/N2	41
Distant metastases	
M0	103
M1	0
TNM stage grouping	
0	4
I	17
II	41
III	41
Lymphovascular invasion	
Positive	55
Negative	43

staged according to the American Joint Committee on Cancer (AJCC) TNM staging system. For participants undergoing colonoscopic examination at the Department of Internal Medicine between 2007 and 2008, blood samples were generally drawn in a fasting state immediately before the procedure. Based on the histological results of colonoscopy, age-matched participants were classified into one of two mutually exclusive categories: normal colonoscopy ($n = 30$) and adenomatous polyps ($n = 33$). In addition, age-matched normal subjects and patients with adenomatous polyps who were enrolled in the study did not have clinical manifestations of chronic diseases such as diabetes, hepatitis, tuberculosis, or autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus at the time of colonoscopic examination.

Preparation of peripheral blood total RNA and cDNA

PBMCs were purified by gradient centrifugation of heparinized blood using Ficoll-Hypaque. Total RNA was extracted using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized by reverse transcription with a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, USA), according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

All of the primers and probes for the B7/CD28 family members and the PCR master mix were purchased from Applied Biosystems (USA). Expression of target genes was measured via qRT-PCR using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The thermal cycling program used for qRT-PCR was as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Gene expression levels were calculated as a relative quantification using standard curves generated by serial dilutions of human cancer cell line cDNAs containing each target gene. All analyses were carried out in duplicate.

Statistical analysis

The expression of B7 and CD28 family PBTs was compared among three subjective groups and between colon cancer patients with or without microscopic LVI using the Mann–Whitney U test. Differences in expression levels of co-signaling molecules across all cancer stages were analyzed using the Kruskal–Wallis test. All statistical investigations were performed using the Statistical Package for the Social Sciences (SPSS version 13.0 for Windows, Chicago, IL, USA) and MedCalc Statistical Software (version 9.6.4.0 for Windows, Mariakerke, Belgium). $P < 0.05$ was considered statistically significant.

Results

Expression analysis of B7/CD28 family PBTs

We first examined the blood transcript levels of each B7/CD28 family member using real-time PCR. Unlike the previous results showing the upregulation of cancer-associated co-inhibitory ligands such as B7-H1, B7-H3, and B7-DC, PBT expression of B7-H3, but not B7-H1 and B7-DC, was significantly higher in colon cancer patients than in normal subjects ($P = 0.028$, Fig. 1). Rather, B7-DC PBTs were downregulated in colon cancer patients compared to normal subjects ($P = 0.044$). However, there was no significant difference in PBT levels of the rest of B7 family ligands between normal subjects and colon cancer patients (Fig. 1). PBT expression of CD28 family receptors were differentially expressed in colon cancer patients; co-stimulatory receptors including CD28 and ICOS were transcribed at a lower level ($P < 0.001$, $P = 0.009$, respectively) and co-inhibitory PD-1 PBT was significantly higher in colon cancer than in normal subjects ($P < 0.001$). Interestingly, unlike PD-1, PBT expression of co-inhibitory receptors such as CTLA-4 and BTLA was significantly lower in colon cancer patients than in normal subjects ($P = 0.010$, $P = 0.002$, respectively). We also compared the PBT expression of B7/CD28 family between patients with adenomatous polyps and normal subjects. There was a significant downregulation of B7-2, B7-DC, CD28, and ICOS and a significant upregulation of PD-1 in patients with adenomatous polyps compared with normal controls. In addition, the result showed a significant upregulation of B7-H3 PBT ($P = 0.013$) and downregulation of BTLA ($P < 0.001$) in colon cancer patients compared to those with adenomatous polyps. Interestingly, B7-H4 PBTs were not detectable after qRT-PCR using Taqman primers and probes for exons three and four of B7-H4 (data not shown).

We also evaluated B7/CD28 family PBTs for discrimination of colon cancer patients from normal subjects using ROC curves. The area under the curve (AUC) values obtained by ROC curve analysis were higher for CD28 family than for B7 family. The average AUC for B7 family versus CD28 family was 0.56 ± 0.06 versus 0.69 ± 0.03 ($P = 0.005$). The highest AUC to identify colon cancer patients was found for BTLA (0.725) followed by CD28 (0.723) and PD-1 (0.703). Interestingly, as shown in Fig. 2, the multivariate logistic regression ROC curve for

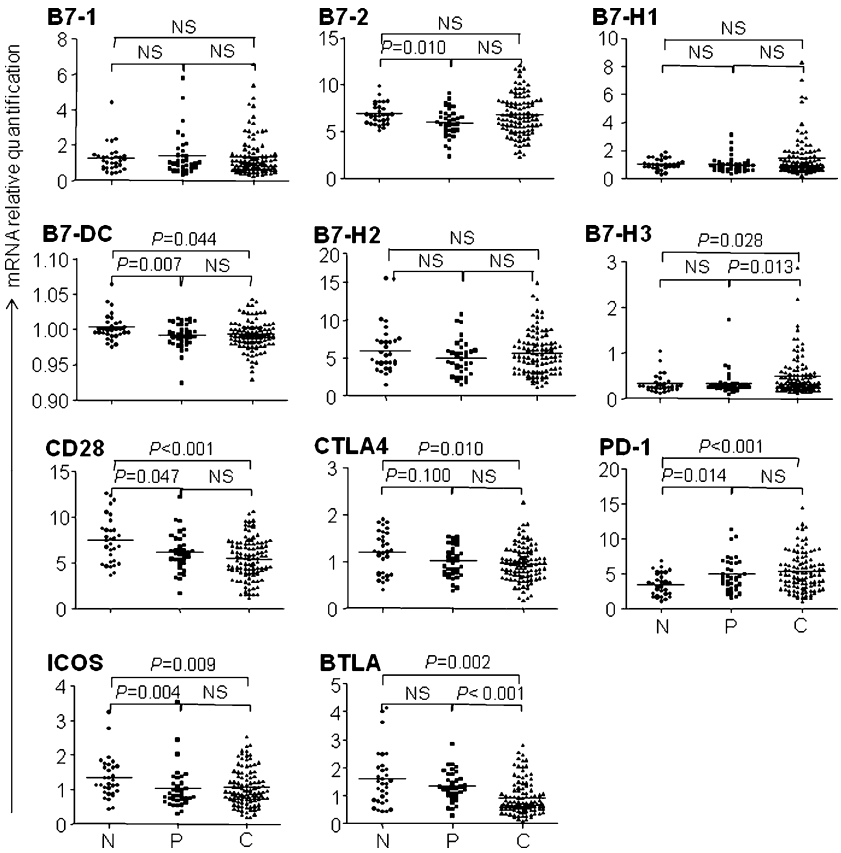

Fig. 1 Expression of peripheral blood transcripts of B7/CD28 family members. Total RNA was extracted from PBMCs of normal subjects and patients with adenomatous polyps or colon cancer. Quantitative real-time PCR for B7/CD28 family was performed. Gene expression levels were calculated as a relative quantification using standard curves generated by serial dilutions of human cancer cell line cDNAs containing each target gene (*N* normal subjects, *P* patients with adenomatous polyps, *C* patients with colon cancer)

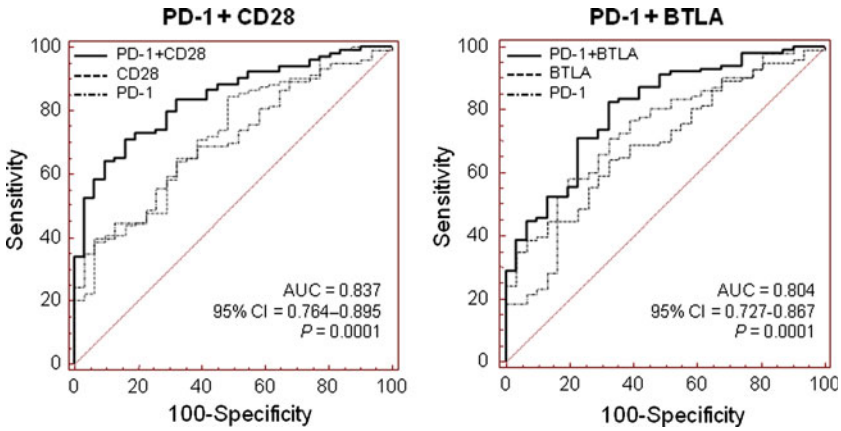

Fig. 2 ROC curve analysis of combined PD-1 and CD28 or BTLA for differentiation of patients with TNM stage I and II colon cancer from normal subjects

combined co-signaling molecule PBTs greatly enhanced AUC values to discriminate colon cancer patients with TNM stage I and II, and normal subjects; PD-1 in combination with CD28 and BTLA PBTs yielded AUC values of 0.837 (95% CI, 0.764–0.895) and 0.804 (95% CI, 0.727–0.867), respectively, significantly higher than that of each PD-1 and CD28 PBT alone. A similar enhancement of AUC value was observed in the analysis of ROC curve for combined co-signaling molecule PBTs in the colon cancer patients with any TNM stages and normal subjects (data not shown).

B7/CD28 family PBTs and primary colon cancer pathology

We compared the PBT expression of B7/CD28 family members with pathologic features of colon cancer including tumor invasion staged according to the 2002 primary tumor classification and AJCC TNM staging system, and lymph node involvement. Among the co-signaling molecules examined, only B7-H3 showed a significant upregulation of PBT with increasing tumor invasion (Table 2); the median B7-H3 PBT value from colon cancer patients with Tis/T1, T2, and T3/T4 stage cancers was 0.188, 0.29, and

Table 2 Associations of the expression levels of peripheral blood transcripts of B7 family with clinical and pathologic features for 103 colon cancer patients

Feature	B7-1		B7-2		B7-H1		B7-DC		B7-H2		B7-H3	
	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*
Age												
>65	1.10 (0.24–6.56)	0.062	6.85 (2.71–11.77)	0.236	1.00 (0.41–5.85)	0.838	0.99 (0.93–1.04)	0.713	5.63 (1.16–13.27)	0.042	0.34 (0.14–2.18)	0.927
≤65	0.87 (0.29–6.59)		6.24 (2.30–12.07)		0.99 (0.20–8.30)		0.99 (0.94–1.04)		4.75 (1.31–10.38)		0.32 (0.12–1.31)	
Sex												
Male	0.92 (0.24–6.59)	0.615	6.41 (2.30–12.07)	0.484	0.99 (0.36–7.07)	0.984	0.99 (0.95–1.04)	0.319	5.06 (1.31–11.29)	0.175	0.35 (0.14–1.31)	0.542
Female	1.07 (0.35–6.56)		6.92 (2.56–11.77)		0.10 (0.20–8.30)		0.99 (0.93–1.04)		5.63 (1.16–13.27)		0.32 (0.12–2.18)	
2002 primary tumor classification												
Tis and T1	1.11 (0.56–3.62)	0.587	6.63 (4.12–11.68)	0.935	1.10 (0.56–2.91)	0.856	0.99 (0.96–1.04)	0.738	6.57 (3.01–13.27)	0.126	0.188 (0.14–1.06)	0.025
T2	1.29 (0.36–6.59)		6.25 (5.06–10.04)		0.90 (0.54–7.07)		0.99 (0.98–1.01)		6.25 (1.84–10.50)		0.29 (0.14–1.03)	
T3 and T4	0.93 (0.24–6.56)		6.77 (2.30–12.07)		0.96 (0.20–8.29)		0.99 (0.93–1.04)		5.08 (1.16–12.81)		0.35 (0.12–2.18)	
Lymph node involvement												
N0	0.99 (0.29–6.59)	0.694	6.49 (2.30–11.77)	0.661	1.01 (0.36–7.07)	0.798	0.99 (0.93–1.04)	0.167	5.96 (1.75–13.27)	0.028	0.30 (0.14–2.18)	0.262
N1/N2	0.93 (0.24–5.39)		6.98 (2.56–12.07)		0.94 (0.20–8.29)		1.00 (0.95–1.03)		4.65 (1.16–9.78)		0.42 (0.12–1.59)	
TNM stage grouping												
0	2.02 (1.11–3.34)	0.316	7.26 (5.06–11.02)	0.681	1.51 (0.99–2.43)	0.59	0.98 (0.96–0.99)	0.136	9.74 (5.44–11.29)	0.031	0.18 (0.14–0.19)	0.019
I	0.87 (0.36–6.59)		6.64 (4.12–11.68)		0.90 (0.54–7.07)		1.00 (0.96–1.04)		6.00 (1.84–13.27)		0.27 (0.14–1.06)	
II	0.93 (0.29–6.56)		6.30 (2.30–11.77)		1.03 (0.36–5.85)		0.99 (0.93–1.04)		5.31 (1.75–12.81)		0.32 (0.17–2.18)	
III	0.93 (0.24–5.39)		6.98 (2.56–12.07)		0.94 (0.2–8.3)		1.00 (0.95–1.03)		4.65 (1.16–9.78)		0.42 (0.12–1.59)	

0.35, respectively ($P = 0.025$). Additionally, the median B7-H3 PBT value from cancer patients with TNM stages 0, I, II, and III also significantly increased to 0.18, 0.27, 0.32, and 0.42, respectively ($P = 0.019$). Elevated B7-H3 PBT level was observed in colon cancer patients with regional lymph node involvement; however, this trend was not statistically significant ($P = 0.262$). Contrast to B7-H3 PBT, the median value of B7-H2 PBT from patients with TNM stage 0, I, II, and III colon cancers significantly decreased to 9.74, 6.0, 5.31, and 4.65, respectively ($P = 0.031$). In addition, a significant decline in the median B7-H2 PBT level was also observed in patients with colon cancer that was extended into regional lymph nodes ($P = 0.028$). The median value of B7-H2 PBT decreased in colon cancer patients with Tis/T1, T2, and T3 cancers, but, the decrease did not reach statistical significance ($P = 0.126$). No clear relationship was observed between the PBT levels of the rest of B7/CD28 family and the pathologic features such as tumor invasion and regional lymph node involvement; however, there was a tendency for CTLA-4 and ICOS PBTs to decrease across all TNM stages (Table 3). B7/CD28 family PBTs did not vary according to age or sex in colon cancer patients with the exception of B7-H2; colon cancer patients over age 65 showed a significant increase in B7-H2 PBT expression compared to the patients at age 65 or less ($P = 0.042$) (Table 2). No statistically significant correlations were observed between B7/CD28 family PBT expression and serum CEA concentrations (data not shown).

B7/CD28 family PBTs and lymphovascular invasion of colon cancer

We next examined whether the co-signaling molecule PBTs discriminate colon cancer patients with LVI ($n = 55$) from those without LVI ($n = 43$). ROC curves used the microscopic invasion into adjacent lymphovasculature as the end point for discrimination. No significant relationship was observed between single PBT level of B7/CD28 family and the presence of microscopic LVI in primary colon cancer patients (data not shown). However, ROC curve analyses showed that the PBT ratios of co-inhibitory receptor to B7 family were able to discriminate colon cancer patients with microscopic LVI from those without LVI. Specifically, the highest AUC to detect colon cancer patients with LVI was found for the ratio of CTLA-4 to B7-H2 (AUC = 0.690, 95% CI = 0.588–0.780) followed by the PBT ratio of PD-1 to B7-H2 (AUC = 0.643, 95% CI = 0.539–0.737), and CTLA-4 to B7-1 (AUC = 0.623, 95% CI = 0.519–0.719). However, ROC curves for the PBT ratios of co-inhibitory receptors other than CTLA-4 and PD-1 to B7 family was not statistically significant (data not shown). Consistent with this finding, the median value of the PBT ratio of CTLA-4 to B7-H2 was significantly

Table 3 Associations of the expression levels of peripheral blood transcripts of CD28 family with clinical and pathologic features for 103 colon cancer patients

Feature	CD28		CTLA4		ICOS		PD-1		BTLA	
	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*	Median (range)	*P*
Age										
>65	5.75 (1.64–10.62)	0.480	0.96 (0.26–1.52)	0.678	0.93 (0.26–2.55)	0.209	4.80 (1.55–11.60)	0.769	0.76 (0.24–2.82)	0.359
≤65	4.88 (1.18–9.67)		0.91 (0.18–2.27)		0.85 (0.19–2.01)		5.02 (1.02–14.48)		0.67 (0.12–2.56)	
Sex										
Male	5.66 (1.18–10.40)	0.655	0.94 (0.18–2.27)	0.808	0.93 (0.27–2.55)	0.714	4.79 (1.02–12.17)	0.507	0.76 (0.12–2.56)	0.834
Female	5.03 (1.57–10.62)		0.94 (0.22–1.81)		0.90 (0.19–2.30)		5.12 (1.88–14.48)		0.69 (0.14–2.82)	
2002 primary tumor classification										
Tis and T1	5.04 (1.64–6.90)	0.453	1.00 (0.26–1.38)	0.996	0.94 (0.38–2.14)	0.610	4.38 (2.62–9.32)	0.551	0.64 (0.35–2.13)	0.70
T2	5.97 (4.09–8.55)		0.91 (0.62–1.48)		1.14 (0.44–2.01)		3.84 (1.80–9.15)		0.76 (0.54–1.89)	
T3 and T4	5.49 (1.18–10.62)		0.93 (0.18–2.27)		0.89 (0.19–2.55)		5.12 (1.02–14.48)		0.72 (0.15–2.82)	
Lymph node involvement										
N0	4.88 (1.18–10.62)	0.334	0.94 (0.18–1.80)	0.609	0.97 (0.26–2.55)	0.228	4.85 (1.02–12.17)	0.644	0.67 (0.12–2.82)	0.405
N1/N2	5.87 (1.57–9.53)		0.87 (0.22–2.27)		0.82 (0.19–2.25)		5.59 (1.55–14.48)		0.80 (0.14–2.56)	
TNM stage grouping										
0	5.14 (4.03–6.60)	0.553	1.05 (0.76–1.31)	0.819	1.00 (0.78–2.14)	0.594	6.29 (3.12–9.32)	0.866	0.92 (0.61–2.13)	0.5
I	5.36 (1.64–8.55)		0.96 (0.26–1.48)		0.98 (0.38–2.01)		4.76 (1.80–9.15)		0.71 (0.34–1.89)	
II	4.5 (1.18–10.62)		0.93 (0.18–1.80)		0.93 (0.26–2.55)		4.90 (1.02–12.17)		0.66 (0.12–2.82)	
III	5.87 (1.57–9.53)		0.87 (0.22–2.27)		0.82 (0.19–2.25)		5.59 (1.55–14.48)		0.8 (0.14–2.56)	

Table 4 Associations of the ratios of CD28 family to B7 family transcripts in colon cancer patients with or without lymphovascular invasion

Gene ratio	Lymphovascular invasion (LVI)[a]		*P* value
	Positive ($n = 55$)	Negative ($n = 43$)	
CTLA-4 to B7-H2	0.22 (0.05–0.92)	0.14 (0.04–0.43)	0.001
PD-1 to B7-H2	1.17 (0.31–4.23)	0.81 (0.19–2.83)	0.016
CTLA-4 to B7-1	1.00 (0.20–3.09)	0.80 (0.18–2.51)	0.038
CTLA-4 to B7-2	0.16 (0.04–0.32)	0.13 (0.04–0.35)	0.043
PD-1 to B7-1	5.87 (1.09–23.5)	4.01 (0.67–14.5)	0.140
PD-1 to B7-2	0.81 (0.24–3.45)	0.57 (0.16–5.29)	0.113

[a] Values are median (range)

higher in LVI-positive colon cancer patients than in LVI-negative patients (0.22 vs. 0.14, $P = 0.001$) (Table 4). We also found that the PBT ratios of PD-1 to B7-H2 and CTLA-4 to B7-1 showed significantly higher median values in colon cancer patients with LVI than in those without LVI (for PD-1 to B7-H2 ratio, 1.17 vs. 0.81, $P = 0.016$; for CTLA-4 to B7-1 ratio, 1.0 vs. 0.8, $P = 0.038$).

Discussion

In this study, we demonstrate the global gene expression of B7/CD28 family blood transcripts in normal subjects and patients with adenomatous polyps and colon cancer. Our results reveal a significant downregulation of co-stimulatory ligands and receptors such as B7-2, B7-DC, CD28, and ICOS and a significant upregulation of co-inhibitory receptor PD-1 in patients with adenomatous polyps compared with normal controls. This result suggests that expression profiles of B7/CD28 family related to an immunosuppressive status is already established even in patients with adenomatous polyps. In addition, the global gene expression profiles also show that blood transcripts of B7-H3 and PD-1, co-inhibitory molecules associated with poor prognosis in cancer patients, are significantly upregulated, whereas transcripts of co-stimulatory receptors including CD28 and ICOS are downregulated in colon cancer patients. This result is in line with the previous studies showing that upregulation of co-inhibitory molecules in tumor tissues or increase of soluble forms of co-inhibitory molecules in peripheral bloods from cancer patients has prognostic values to predict clinical outcomes in patients with various cancers including renal carcinoma (Thompson et al. 2006), pancreatic cancer (Loos et al. 2008), esophageal carcinoma (Ohigashi et al. 2005), and ovarian cancer (Simon et al. 2007). Therefore, our data suggest that blood transcript of co-signaling molecules in colon cancer patients can reflect the expression profile of cancer-associated co-signaling molecules in peripheral tumor tissues. This notion can be explained by the previous reports that cytokines from tumor microenvironment affect the expression of co-inhibitory molecules on tumor cells; cancer-associated B7-H1 is upregulated by IL-10 and VEGF (Curiel et al. 2003), and B7-H4 expression on tumor macrophages is increased by IL-6 and IL-10 (Kryczek et al. 2006). Thus, it is reasonable to speculate that blood transcripts of B7/CD28 family in cancer patient immune cells may be differentially regulated by the tumor-derived cytokines, reflecting expression patterns of co-signaling molecules in tumor microenvironment. Further study is needed to validate the correlation of blood transcript expression with tissue expression of co-signaling molecules in cancer patients. Our results also demonstrate that combining two different co-signaling molecule PBTs greatly improves the ability to differentiate patients with early stage colon cancer from normal subjects. For example, combination of PD-1 and CD28 PBTs is more likely to discriminate patients with TNM stage I and II colon cancer from normal subjects compared to each PBT alone. However, due to overlapping PBT expression between normal subjects and patients with adenomatous polyps or colon cancer, it is unlikely that each B7 and CD28 PBT would be an effective screening method for detection of the presence of colon cancer and adenomatous polyps.

Our results also indicate that B7 rather than CD28 family PBTs are associated with tumor progression. Specifically, PBT expression of B7-H2, a co-stimulatory ligand, is significantly downregulated in colon cancer patients with lymph node involvement compared with those without lymph node metastasis. Furthermore, a significant decrease of B7-H2 blood transcript is seen in patients with advanced colon cancer stage compared with the patients with early stage of colon cancer. Interestingly, although B7-H2 PBT profile does not discriminate colon cancer patients from normal subjects; its expression in colon cancer patients is negatively associated with pathological features, including regional lymph node metastasis and aggressive tumor progression. For some reasons, B7-H2 blood transcript is increased in old colon cancer patients over age 65. In contrast, PBT level of B7-H3, a co-inhibitory ligand associated with poor prognosis of cancer patients, shows a strong association with more aggressive tumor invasion and advanced TNM stage, as evidenced by a significant upregulation of B7-H3 PBT in colon cancer patients with increased tumor invasion or advanced TNM stage compared with the patients with Tis/T1 and T2 primary tumor classification or early TNM stage. Although PBT expression of PD-1 is significantly upregulated in colon cancer patients, it is not associated with colon cancer progression. Therefore, co-signaling ligand PBTs, such as B7-H2 and B7-H3, may serve as valuable markers reflecting the

pathological features of colon cancer. Despite inability of each PBT to discern LVI-positive colon cancer patients from LVI-negative patients, the ratios of PBT expression of CD28 family to B7 family members, especially those of CTLA-4 to B7-H2 and PD-1 to B7-H2, enables discrimination of patients with microscopic LVI-positive cancers from those with LVI-negative cancers, indicating that the PBT ratios, rather than each PBT alone, of B7/CD28 family have a significant predictive value for cancerous microscopic invasion. Thus, blood transcripts of B7/CD28 family may be complementary to immunohistochemical analysis for the pathological features of cancer in the context of predictive measure able to reflect the cancer progression.

To our knowledge, this study is the first to report on the relationship between B7/CD28 family blood transcripts and the pathological features of colon cancers, such as aggressive tumor progression and microscopic LVI. The major shortcomings of our study include the relatively small number of peripheral blood samples examined and uneven TNM cancer stage distribution among patients. Nevertheless, we found strong associations between the expression of B7/CD28 family transcripts and the pathological features in colon cancer patients. A long-term follow-up study is needed to determine whether B7/CD28 family PBT expression is correlated with clinical outcome.

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Conflict of interest statement None.

References

Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH et al (2006) Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439:682–687

Boorjian SA, Sheinin Y, Crispen PL, Farmer SA, Lohse CM, Kuntz SM et al (2008) T cell coregulatory molecule expression in urothelial cell carcinoma: clinicopathologic correlations and association with survival. Clin Cancer Res 14:4800–4808

Choi IH, Zhu G, Sica GL, Strome SE, Cheville JC, Lau JS et al (2003) Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. J Immunol 171:4650–4654

Crispen PL, Sheinin Y, Roth TJ, Lohse CM, Kuntz SM, Frigola X et al (2008) Tumor cell and tumor vasculature expression of B7-H3 predict survival in clear cell renal cell carcinoma. Clin Cancer Res 14:5150–5157

Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P et al (2003) Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat Med 9:562–567

Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB et al (2002) Tumor-associated B7-H1 promotes T cell apoptosis: a potential mechanism of immune evasion. Nat Med 8:793–800

Greenwald RJ, Freeman GJ, Sharpe AH (2005) The B7 family revisited. Annu Rev Immunol 23:515–548

Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M et al (2005) Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res 65:1089–1096

Jeong HY, Lee YJ, Seo SK, Lee SW, Park SJ, Lee JN, Choi I et al (2008) Blocking of monocyte-associated B7-H1 (CD274) enhances HCV-specific T cell immunity in chronic hepatitis C infection. J Leukoc Biol 83:755–764

Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M (2004) B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. Clin Cancer Res 10:5094–5100

Kryczek I, Zou L, Rodriguez P, Zhu G, Wei S, Mottram P et al (2006) B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. J Exp Med 203:871–881

Loos M, Giese NA, Kleeff J, Giese T, Gaida MM, Bergmann F et al (2008) Clinical significance and regulation of the costimulatory molecule B7-H1 in pancreatic cancer. Cancer Lett 268:98–109

Ohigashi Y, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N et al (2005) Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res 11:2947–2953

Prasad DV, Nguyen T, Li Z, Yang Y, Duong J, Wang Y et al (2004) Murine B7-H3 is a negative regulator of T cells. J Immunol 173:2500–2506

Roth TJ, Sheinin Y, Lohse CM, Kuntz SM, Frigola X, Inman BA et al (2007) B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. Cancer Res 67:7893–7900

Simon I, Katsaros D, Rigault de la Longrais I, Massobrio M, Scorilas A, Kim NW et al (2007) B7-H4 is over-expressed in early-stage ovarian cancer and is independent of CA125 expression. Gynecol Oncol 106:334–341

Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS et al (2006) Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 66:3381–3385

Wang L, Fraser CC, Kikly K, Wells AD, Han R, Coyle AJ et al (2005) B7-H3 promotes acute and chronic allograft rejection. Eur J Immunol 35:428–438

Wu CP, Jiang JT, Tan M, Zhu YB, Ji M, Xu KF et al (2006) Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis. World J Gastroenterol 12:457–459

Yao S, Chen L (2006) Reviving exhausted T lymphocytes during chronic virus infection by B7-H1 blockade. Trends Mol Med 12:244–246

Zang X, Thompson RH, Al-Ahmadie HA, Serio AM, Reuter VE, Eastham JA et al (2007) B7-H3 and B7x are highly expressed in human prostate cancer and associated with disease spread and poor outcome. Proc Natl Acad Sci USA 104:19458–19463