B7 molecule mRNA expression in colorectal carcinoma

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AIM: To observe the status of tumor-associated B7 molecule mRNA expression in human colorectal cancer tissue by in situ hybridization.

METHODS: The mRNA expression patterns of cancer-associated B7.1, B7H1, B7H2, ICOS in 22 specimens of human colorectal cancer tissue were monitored by in situ hybridization (ISH) with digoxin-labeled oligonucleotide probes.

RESULTS: B7.1, B7H1, B7H2, ICOS mRNA were detected in both cancer cells and tumor infiltrating lymphocytes (TIL). The mRNA expression level of these molecules in tumor cells was higher than that in TIL (0.76±0.54-1.62±0.82 vs 0.38±0.19-0.65±0.33, P<0.001). There was no relationship between expression level of tested B7 family molecules and patients’ sex, age, differentiation status of cancer and regional lymph node metastasis.

CONCLUSION: Th2 cytokine predominant in tumor microenvironment might be related to the expression of B7H1, B7H2 co-signal molecules in tumor cells and TIL. Elucidation of tumor-associated B7 molecules may contribute to the design of T cell-based cancer immunotherapy.

Key words: Colorectal cancer; B7.1, B7H1, B7H2 and ICOS; Tumor immunity; Immune evasion

Abstract

The mRNA expression patterns of cancer-associated B7.1, B7H1, B7H2, ICOS in 22 specimens of human colorectal cancer tissue were monitored by in situ hybridization (ISH) with digoxin-labeled oligonucleotide probes. The mRNA expression level of these molecules in tumor cells was higher than that in TIL (0.76±0.54-1.62±0.82 vs 0.38±0.19-0.65±0.33, P<0.001). There was no relationship between expression level of tested B7 family molecules and patients’ sex, age, differentiation status of cancer and regional lymph node metastasis.

CONCLUSION: Th2 cytokine predominant in tumor microenvironment might be related to the expression of B7H1, B7H2 co-signal molecules in tumor cells and TIL.

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INTRODUCTION

Although it has been well accepted that human tumor is immunogenic, most patients suffering from cancer are destined to die due to tumor progress. Establishment of the bisignal model for T-cell activation leads people to think that inactivation of infiltrating immune potential cells in tumor tissue might be immune anergic owing to the deficiency of co-stimulatory molecules. Tumor cells modified with B7 co-stimulatory molecule gene could be rejected by tumor-bearing host[1,2], but most human cancer tissues express co-stimulators[3,4], suggesting that co-stimulatory molecules might not be the only mechanism of immune evasion. The tumor-associated B7-H1 and B7-H2, new members of B7 family can preferentially stimulate the production of IL-10, promote activated T cell apoptosis[5].

In the present study, the status of tumor-associated B7 molecule mRNA expression in human colorectal cancer tissue was observed using B7-H1, B7-H2 and ICOS cDNA probe, in situ hybridization. The results showed that B7.1, B7-H1, B7-H2, ICOS mRNA were expressed in both cancer cells and tumor infiltrating lymphocytes (TIL), indicating that Th2 cytokine predominant in tumor microenvironment might be related to the expression of B7-H1, B7-H2 co-signal molecules in tumor cells and TIL. Elucidation of tumor-associated B7 molecules may contribute to the design of T cell-based cancer immunotherapy.

MATERIALS AND METHODS

Samples

Tissue samples were obtained from 22 patients with colorectal cancer. The specimen were fixed in 4 g/L formaldehyde in phosphate-buffered saline (PBS) immediately and embedded in paraffin. Serial sections (5 μm in thickness) were cut for in situ hybridization or histological evaluation, and mounted on slides covered with APES, dried overnight at 65 °C, stored at -70 °C until use. The diagnosis of colorectal carcinoma was histopathologically verified in all cases. None of the patients had previously received radio-, chemo-, or immunotherapy.

Preparation of oligonucleotide probes

Primer 3 software was used to design oligonucleotide probes complementary to the mRNA of all kinds of target sequences including B7.1, B7-H1, B7-H2, ICOS mRNA. The specificity of all oligonucleotide probes was analyzed by BLAST software (www.ncbi.nlm.gov). The probes were labeled by tailing the oligonucleotides with digoxigenin-11-dUTP kit (Boehringer Mannheim, German). A labeling activity of 1.56 nmol/L was obtained (Primer3 http://wwwgenome.wi.mit.edu/cgi-bin/primer/Primer3BLAST

http://www.ncbi.nlm.gov/cgi-bin/BLAST). Sequence of oligonucleotide probes of co-stimulatory molecules was tested B7.1: 5’-CAT GAA GCT GTG TGT GGT -3'; B7-H1;
5'-TGCTCGTCCAGATTGCTCTTCGGAGTGAACCTGCGCATACAGTCGAGGCTCCTCCTCTCCCTCACAGTCGACCTGGCACAACAGTGGTGAGTGGTGAGTGGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGTGAGT

**In situ hybridization (ISH)**

Diethyl pyrocarbonate (DEPC) water was used for all solutions necessary for ISH. The sections were deparaffinized in xylene and rehydrated in descending ethanol, followed by digestion with 1 g/L proteinase K at 37°C for 30 min and terminated with 20 g/L glycine in PBS for 5 min. Then, the sections were refixed in 40 g/L polyformaldehyde for 20 min, washed with PBS for 10 min, treated with 0.2 mol/L HCl for 10 min and washed with DEPC water for 3 min. The sections were dehydrated in ascending gradient ethanol, air-dried and followed by prehybridization at 42°C for 2 h. Hybridization reaction was carried out at 42°C for 22 h. Then the sections were washed with gradient SSC thoroughly and followed by treatment with digoxin antibody at 37°C for 2 h. The color was developed in NBT-BCIP substrate, then counterstained with 10 g/L methyl green in distilled water, dehydrated and mounted with neutral gum.

For negative controls, probes and antibodies were replaced by PBS or the slides were treated with RNase A (20 mg/L) at 37°C for 30 min. B7-1 mRNA expression in mononuclear leucocytes was used as positive control.

**Evaluation of results**

Purple blue precipitation in cytoplasm under light microscope was considered as positive signal, and 5 high power fields were randomly chosen from each slide. The percentage of positive cells and the positive cell index (total positive granule number/positive cell number) were calculated. Intensity of the color reaction was classified into 4 grades: strongly positive (+++), positive (++), weakly positive (+) and negative (–) and scored as 3, 2, 1 and 0, respectively. The Accumulation index was determined as percentage of positive cells multiply intensity score.

**Statistics analysis**

All datas were expressed as mean±SD. Analysis was performed by using chi-square test and Spearman correlation analysis with SPSS 11.0 software. *P*<0.05 was considered statistically significant.

**RESULTS**

Expression of B7-1, B7H1, B7H2, ICOS mRNA was detected in both cancer cells and tumor infiltrating lymphocytes (Figure 1). The mRNA expression level of these molecules in tumor cells was higher than that in TIL. (0.76±0.54-1.62±0.82 vs 0.38±0.19-0.65±0.33, *P* = 0.000). However, when the expression of B7 family mRNA was analyzed with respect to the tested members, there was a significant difference in B7H1 and ICOS expression between TIL and cancer cells (*P*<0.05). The expression of B7H1 and ICOS mRNA was higher in tumor cells and TIL. The expression of B7 family molecules either in tumor cells or in TIL was not correlated with patients’ sex, age, differentiation status of cancer and regional lymph node metastasis. B7-H1 expression in TIL and tumor invasiveness was significantly associated with the intensity and the depth of tumor invasion (*P* = 0.050, Tables 1-3).

![Figure 1](image_url) mRNA expression of B7-1 (A), B7H1 (B), B7H2 (C) and ICOS (D) in human colorectal cancer tissues (ISH×400).

**Table 1** B7 family molecule expression in colorectal cancer cells and TIL (mean±SD)

| B7 molecule | Tumor cell (mean±SD) | TIL (mean±SD) |
|-------------|----------------------|---------------|
| B7-1        | 1.29±0.63            | 0.45±0.28     |
| B7H1        | 1.38±0.71            | 0.65±0.33     |
| B7H2        | 0.76±0.54            | 0.38±0.19     |
| ICOS        | 1.62±0.82            | 0.53±0.27     |

*a P*<0.05 vs tumor cell.

| B7 molecule | Tumor cell (mean±SD) | TIL (mean±SD) |
|-------------|----------------------|---------------|
| B7-1        | 1.22±0.82            | 0.36±0.43     |
| B7H1        | 1.43±0.63            | 0.64±0.25     |
| B7H2        | 0.62±0.05            | 0.42±0.21     |
| ICOS        | 1.59±1.14            | 0.48±0.35     |

*b P*<0.05 vs T2, T4.
function and also plays an important role in acquired immunity[8,10]. This could well elucidate our previous findings, i.e. although expression of B7-1 by human tumor cells can activate T cells and enhance the secretion of Th1 cytokines such as IL-2 and IFN-γ, Th2 cytokine predominates ultimately since co-stimulators such as B-H1, B-H2 and ICOS act dominantly.

In the present study, we observed that B7-1 co-stimulatory molecule mRNA was expressed in tumor cells and tumor infiltrating lymphocytes (TIL) of human colorectal carcinoma by in situ hybridization, which is consistent with many experiments of B7-gene transfection vaccine[11,12]. However, the other members of B7 family such as B-H1, and ICOS can also be detected. In our study, the expression of B7-molecules in tumor cells was higher than that in TIL (P<0.005), and B-H1 and ICOS mRNA expression in tumor cells was even higher than that of B7-1, suggesting that B7-family plays a role in tumor immunity. In addition, the expression of B-H1 and ICOS mRNA is related with the invasion depth of tumor, the mRNA expression of B-H1, and ICOS in both tumor cells and TIL is associated with the metastasis of colorectal carcinoma. A recent study on B7-family molecules in tumor biological behavior demonstrated that aberrant expression of B-H1 in renal cell carcinoma apparently impairs T cell function and survival[13]. Our results and these data provide the morphological and clinical support to elucidate the role of B7-co-stimulatory molecules in tumor immune evasion.

The present findings indicate that new members of B7 family such as B-H1, B-H2 and ICOS are involved in promoting TH2-based responses preferentially. More interestingly, it has been reported that tumor-associated B-H1 can promote apoptosis of antigen-specific human T-cell clones in vitro[14,18], and mouse P815 tumor-expressed B-H1 increases apoptosis of activated tumor-reactive T-cells and promotes the growth of highly immunogenic B7-1 tumor in vivo[9,10]. These results suggest that induction of apoptosis of tumor-infiltrating lymphocytes by B-H1 molecules overexpressed in human colorectal carcinoma may be a potentially active escape strategy of various tumors from immune attack as is shown by Wintterle et al.[18].

Tumor escape is attributed to a variety of immune evasion strategies, including downregulation of MHC-I class molecules, regulatory T cells, Th2 cytokines, secretion of immunosuppressive factors from tumor cells or TIL, and lack of T-cell co-stimulation. Tumor local microenvironment plays an essential role in determining the final destiny of antitumor immunity[17].

The discovery of new members of B7-family presenting different co-stimulatory effects indicates that different co-signaling molecules lead to different results, such as enhancement of T-cell Receptor (TCR)-mediated immune responses or inhibition of TCR-mediated immune responses. Tumor-associated B-H1 might produce tumor immune escape by promoting apoptosis of tumor-reactive T-cells and type II cytokine secretion through PD-1 ligand[18,19]. Hirano et al.[20], reported that B-H1/PD-1 forms a molecular shield to prevent destruction by CTL, suggesting that to block B-H1 or PD-1 by specific monoclonal antibodies could reverse this resistance and profoundly enhance therapeutic efficacy. Strome et al.[21], have shown the
feasibility of the new immunotherapy. With the suggestion of the concept of co-inhibitors\textsuperscript{3,25}, investigation of the functional characteristics of tumor-associated signal molecules may contribute to exploitation of T cell-based tumor immunotherapy.

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