

RESEARCH ARTICLE

B7-H4 Expression is Associated with Cancer Progression and Predicts Patient Survival in Human Thyroid Cancer

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Abstract

Objective: This study aimed to investigate the expression of B7-H4 in human thyroid cancer and determine any association with patient clinicopathological parameters and survival. **Methods:** B7-H4 expression in 64 clinical thyroid cancer specimens was assessed with immunohistochemistry. Moreover, B7-H4 mRNA expression in 10 fresh resected specimens were evaluated by the reverse transcription-polymerase chain reaction (RT-PCR). Immunohistochemical staining of CD3 was performed to assess the number of tumor infiltrating T lymphocytes (TILs) in thyroid cancers. **Results:** Positive B7-H4 immunohistochemical staining was observed in 61 out of 64 (95.3%) specimens of thyroid cancer tissues. Significantly more B7-H4 mRNA copies were found in thyroid cancer tissue than that adjacent normal tissue. Moreover, B7-H4 expression in human thyroid cancer tissues was significantly correlated with patient TNM stages and extrathyroidal extension ($P < 0.05$), being inversely correlated with the number of TILs ($P < 0.05$). The overall survival rate of the patients with higher B7-H4 expression was significantly worse than that of the patients with lower B7-H4 expression. **Conclusions:** This present study suggests that high B7-H4 expression is associated with cancer progression, reduced tumor immunosurveillance and worse patient outcomes in human thyroid cancer.

Keywords: Thyroid cancer - B7-H4 - progression - prognosis - tumor infiltrating lymphocytes

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Introduction

Thyroid cancer is a malignant thyroid neoplasm originating from follicular or parafollicular thyroid cells (Xing, 2013). In recent decades, thyroid cancer is a common endocrine malignancy that has rapidly increased in global incidence (Jemal et al., 2011), and it now is increasing faster than other malignancy (Xie et al., 2012; Siegel et al., 2013). Although the death rate of thyroid cancer is relatively low, the rate of disease recurrence is high, which is associated with increased patient morbidity and mortality (Tuttle et al., 2010). Certainly, a greater understanding of the molecular mechanisms that lead to the formation of thyroid cancer is needed if better methods of prevention, detection, and treatment are to be developed.

The B7-H4 coregulatory molecule is a member of the B7 family of molecules, which play a central role in the regulation of antigen-specific T cell-mediated immune responses. B7-H4, also referred to as B7x or B7S1, is a ligand within the B7 family that has been implicated as a negative regulator of T cell-mediated immunity. Robust B7-H4 protein expression is primarily restricted to activated T cells, B cells, dendritic cells, and monocytes.

Weak expression of B7-H4 has also been observed in some peripheral tissues, presumably surveyed from human autopsy specimens or tissues that were removed because of pathologic conditions not directly involving the organ being examined. Recently, B7-H4 has been reported to be highly expressed in human cancer cells, and their protein levels in tumors significantly correlate with patients' clinicopathological features and postoperative prognosis (Miyatake et al., 2007; Awadallah et al., 2008; Arigami et al., 2011; Abadi et al., 2013). However, no reports have investigated the clinical significance of B7-H4 expression in patients with thyroid cancer.

In the present study, we investigated tumor microenvironment by examining B7-H4 protein expression as well as densities of various TIL subsets in human thyroid cancer tissues using the immunohistochemical method. B7-H4 expression in relation to patients' clinicopathological parameters and densities of TILs was analyzed. Prognostic values of B7-H4 were evaluated in a log-rank survival analysis. Our results showed that expression levels of the inhibitory costimulatory molecule B7-H4 in human thyroid cancer tissues were significantly correlated with cancer progression, densities of TILs, and poorer patient outcome.

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Materials and Methods

Patient population

Formalin-fix, paraffin-embedded tumor tissue blocks were collected retrospectively from the Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine. All of the 64 thyroid cancer patients underwent surgical resection between December 2006 and August 2008 in Xinhua hospital. None of the patients received chemotherapy or radiotherapy before surgery. The pathologic reports were reviewed, and the tumor–node–metastasis (TNM) stages were assigned according to the American Joint Committee on Cancer staging system. Patients' clinical parameters are shown in Table 1, and patients' survival intervals are dated toward the end of December 2012. In addition, 8 normal tissues from the non-malignant portion of thyroid were resected from surgery and used as controls. Ethical approval for this study was granted by the ethics committee of Xinhua hospital and informed consents were obtained.

Immunohistochemical staining

B7-H4 expression was analyzed using human thyroid cancer and normal tissue. Standard immunohistochemical procedures were carried out using VECTASTAIN Elite ABC system (Vector Laboratories, USA), according to the manufacturer's protocol. Anti-B7-H4 monoclonal antibody (Abcam, USA) was used as a primary antibody. Similar tissue sections were immunostained with normal IgG were used as negative controls. In the immunohistochemical procedure for CD3, anti-human CD3 antibody (DAKO, USA) diluted 1:100 in PBS was used. The specimens were examined and scored using a two-headed microscope. Staining intensity (0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining) and the proportion of stained cells (0, no staining; 1, <10% staining; 2, between 11% and 33%; 3, between 34% and 66%; and 4, >67%) were semi-quantitatively determined. The intensity and percentage of positive cell scores were multiplied (0-12). All slides were scored by two observers blinded to the pathology and clinical features. There are good concordances between two observers. In cases where score difference was equal to or exceeding 2, the slides were re-examined and a consensus was reached by the observers. Mean score for duplicate cores from each individual was calculated.

Quantitative RT-PCR

Fresh tumor specimens were homogenized in FastPrep (Qbiogene, USA). Quantitative real-time PCR (qRT-PCR) analysis was carried out to detect the expression of B7-H4 in 10 human thyroid cancer tissues and corresponding adjacent normal tissues. Total RNA extraction from thyroid cancer tissue was performed with Trizol Reagent (Invitrogen). Then, 2 µg of total RNA was reverse-transcribed with Taqman (TakaRa PrimeScript™ RT Reagent Kit, Japan). The primers below were used to amplify B7-H4 and GAPDH. B7-H4: (Forward), 5'-CTTCTGCCTCTCAGCCCTTA-3'; (Reverse), 5'-GAAATAGTTCTGTAGATCC CTGTTG-3'; GAPDH: (Forward), 5'-GGGTGTGAACCATGAGAAGT-3';

(Reverse), 5'-GACTGTGGTCATGAGTCCT-3'. Each reaction had a total volume of 25 µl, with reagents from the TaKaRa Ex Taq® kit. Thermocycling conditions included 40 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 15 s and extension at 72°C for 60 s. Data from the array were normalized to GAPDH.

Statistical analysis

All statistics were performed with the GraphPad Prism version 5.0 for Windows (GraphPad Software, USA). The χ^2 test was used to assess the statistical significance of differences between the various tissue specimens. Correlation between variables was evaluated by Spearman's rank correlation coefficients. Survival analysis was carried out using the Kaplan-Meier method and log-rank test. Differences were considered to be statistically significant at $P < 0.05$.

Results

B7-H4 expression in human thyroid cancer tissues

The B7-H4 expression in 64 tissue specimens obtained from patients with thyroid cancer was assessed by immunohistochemical staining. Interobserver agreement in the assessment of immunohistochemical findings was excellent. Positive B7-H4 immunohistochemical staining was predominantly observed on the membrane and in cytoplasm of thyroid cancer cells (Figure 1), while weak staining was found in normal thyroid tissues. 61 out of 64 (95.3%) specimens of thyroid cancer tissues showed positive B7-H4 staining. Therefore, higher B7-H4 expression was identified in 46 (71.9%) of 64 thyroid cancer specimens. In this study, B7-H4 expression in 20 fresh tumor specimens from thyroid cancer patients was also assessed with the RT-PCR assay. In clinical thyroid tissues, B7-H4 mRNA expression was confirmed in all of tumor specimens and higher B7-H4 mRNA expression was found in tumor tissues than that in adjacent normal tissues ($P < 0.01$) (Figure 2).

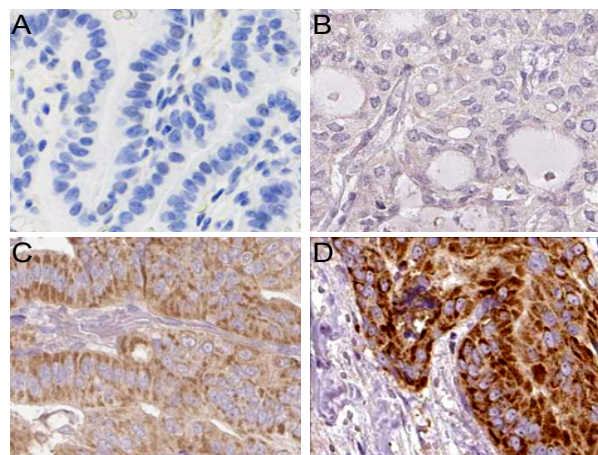


Figure 1. Representative Immunohistochemical Staining of B7-H4 Expression in Thyroid Cancer Tissues. Tumor cells with negative (A), weak (B), moderate (C), and strong (D) expression of B7-H4. Immunohistochemistry showed that positive B7-H4 immunochemical staining was predominantly observed on the membrane and in cytoplasm of tumor cells. Original magnification $\times 400$.

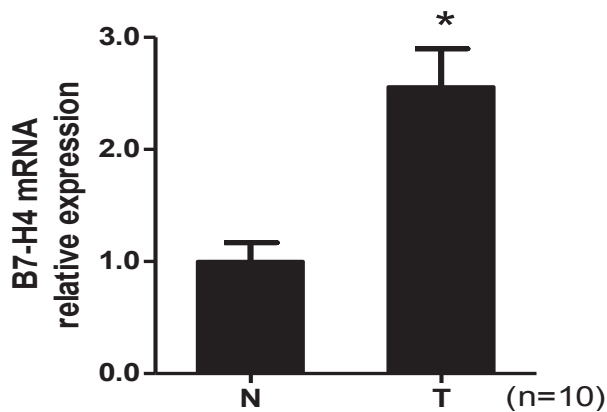


Figure 2. Higher B7-H4 mRNA Expression in Clinical Thyroid Cancer Tissues. Quantification of RT-qPCR for B7-H4 mRNA expression in 10 matched human tissues was conducted using paired t test and bars correspond to mean \pm SD (* $P < 0.05$). The relative B7-H4 mRNA expression was standardized to GAPDH

Table 1. B7-H4 Expression and Correlation with Clinical Parameters in Human Thyroid Cancer

Clinical parameters	Cases	B7-H4 expression		P value
		Group high ^a	Group low ^b	
Gender				0.58
Male	25	17 (68%)	8 (32%)	
Female	39	29 (74%)	10 (26%)	
Age				0.40
<55	24	19 (79%)	5 (21%)	
≥ 55	40	27 (68%)	13 (32%)	
Tumor size (cm)				0.39
<1	19	12 (63%)	7 (37%)	
≥ 1	45	34 (76%)	11 (24%)	
TNM stage				0.01
I	30	17 (57%)	13 (43%)	
II, III, IV	34	29 (85%)	5 (15%)	
Lymph node metastasis				0.08
Yes	22	19 (86%)	3 (14%)	
No	42	27 (64%)	15 (36%)	
Extrathyroidal extension				0.03
Yes	16	15 (94%)	1 (12%)	
No	48	31 (67%)	17 (33%)	

^aB7-H4 staining score > 6 ; ^bB7-H4 staining score ≤ 6

Correlation between B7-H4 expression and patient's clinicopathological features

To further investigate the clinical relevance of B7-H4 protein levels in the thyroid cancer tissues, we categorized 64 patients into two major subgroups according to the intensity of B7-H4 immunohistochemical staining, i.e., the lower B7-H4 expression group (score ≤ 6 , 18 cases), and the higher B7-H4 expression group (score > 6 , 46 cases). The correlation between tumor cell B7-H4 expression and patients' clinicopathologic parameters was shown in Table 1. It demonstrated that B7-H4 expression in human thyroid cancer tissues was significantly correlated with patient's TNM stage ($P < 0.05$) and extrathyroidal extension ($P < 0.05$), whereas it is not correlated with patient's age, gender, tumor sizes, and lymph node metastasis. It is worth mentioning that there was a trend toward a positive correlation between B7-H4 expression and lymph node metastasis ($P = 0.08$). Thus, our data demonstrated that the level of B7-H4 protein was positively correlated with

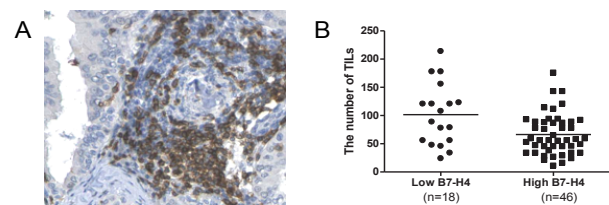


Figure 3. Correlation Between B7-H4 Expression Status and the Number of Tumor Infiltrating T Lymphocytes in Thyroid Cancer. (A) Representative CD3 immunohistochemical staining for the assessment of tumor infiltrating T lymphocytes in thyroid cancer foci. Tumor infiltrating T lymphocytes were diffusely identified in tumor foci. Original magnification $\times 200$. (B) The B7-H4 expression status was inversely correlated with the number of tumor infiltrating T lymphocytes ($P < 0.01$). Horizontal bars indicate the mean number of tumor infiltrating T lymphocytes

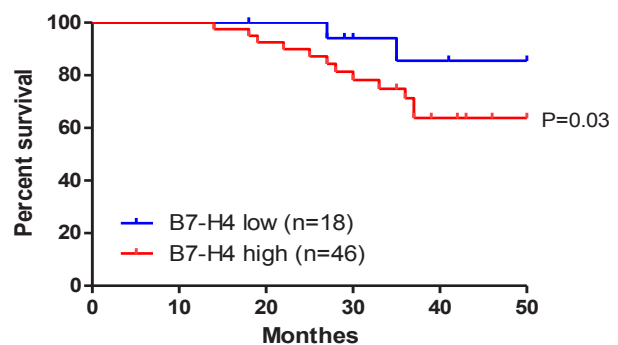


Figure 4. Kaplan-Meier Survival Curves for Thyroid Cancer Patients Based on B7-H4 Expression. Patients with high B7-H4 expression had a significantly poorer prognosis than those with low B7-H4 expression ($P < 0.05$)

advanced clinicopathological parameters and supported that B7-H4 was involved in the progression of human thyroid cancer.

Correlation between tumor cell B7-H4 expression and densities of TILs

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. TILs stained by CD3 antigen were diffusely identified in tumor foci and stroma (Figure 3A). The number of TILs was significantly lower in thyroid cancer patients with high B7-H4 expression (66.4 ± 36.2) than those in patients with low B7-H4 expression (101.7 ± 54.7) ($P < 0.01$) (Figure 3B). Consequently, the B7-H4 expression status of thyroid cancer cells was inversely correlated with the number of tumor infiltrating T lymphocytes ($P < 0.01$).

High B7-H4 expression associated with poor prognosis in human thyroid cancer

We then evaluated the association between B7-H4 expression and survival following radical cystectomy in thyroid cancer patients. Our data showed that higher expression of B7-H4 is associated with a poor outcome following radical cystectomy (Figure 4). The survival rates were significantly lower in patients with high B7-H4 expression than in those with low B7-H4 expression ($P < 0.05$). Our studies support the utility of B7-H4 as

prognostic biomarkers for recurrence in thyroid cancer patients undergoing cystectomy.

Discussion

B7-H4 is a member of the B7 gene family, which has been implicated in negatively regulating T cell mediated immunity. Here, we demonstrated that B7-H4 protein was overexpressed in human thyroid cancer tissues but weakly expressed in normal tissues. We also clarified the clinical significance of B7-H4 expression in thyroid cancer by investigating relationships between B7-H4 protein expression and clinicopathological factors including prognosis. We demonstrated that higher B7-H4 expression was positively correlated with advanced TNM stage and poor patient outcome, implicating its role in thyroid cancer progression. To our knowledge, this study is the first to assess B7-H4 expression and to explore its clinical significance in thyroid cancer. We believe that, with further investigation, B7-H4 could become a clinical prognostic marker and a target for immunotherapeutic treatment of human thyroid cancer.

The costimulatory B7 family members are cell-surface protein ligands, binding to receptors on lymphocytes to regulate immune responses (Flies et al., 2007). They not only provide positive signals to stimulate T-cell activation, but also regulate negative signals to inhibit T-cell responses. Inhibitory B7 molecules have been demonstrated to be upregulated in different tumors, which may contribute to tumor immune evasion (Chen, 2004). B7-H4, a member of the B7 family, has been identified as a negative regulatory molecule on the cell membrane, which inhibits the proliferation and cytokine production of CD4+ T and CD8+ T cells (Prasad et al., 2003; Sica et al., 2003). In the tumor microenvironment, B7-H4 binds to an unknown receptor on the T cell surface, inhibiting tumor-specific T cell activation and proliferation. Accumulated evidences has been shown that increased B7-H4 expression is involved in shaping the tumor microenvironment, and aberrant B7-H4 expression is associated with various clinicopathological features in many human malignancies (Zheng et al., 2012). In gastric cancer, B7-H4 significantly correlated with depth of tumor invasion, lymph node metastasis, and overall stage in gastric cancer (Arigami et al., 2010). B7-H4 expression was significantly higher in invasive breast cancer cells and increased B7-H4 expression was associated with negative progesterone receptor status in breast cancer patients (Tringler et al., 2005). 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 and gastric cancer patients (Oikonomopoulou et al., 2008; Arigami et al., 2010). In the present study, primary thyroid tumor cells displayed various degrees of B7-H4 expression, and its expression was observed in 95.3% patients with thyroid cancer. These results indicate that the majority of patients with thyroid cancer express B7-H4 and that it may be a useful diagnostic marker for patients with thyroid cancer.

In this study, we showed that B7-H4 expression in human thyroid cancer tissues was significantly correlated

with patient's TNM stage and extrathyroidal extension. Although no significant relationship was detected in the statistical analysis, thyroid cancer patients with high B7-H4 expression tended to display the presence of lymph node metastasis compared with those with low B7-H4 expression. Our findings indicated a close relationship between B7-H4 expression and tumor progression in thyroid cancer. Furthermore, patients with high B7-H4 expression had a poorer prognosis compared with those with low B7-H4 expression in human thyroid cancer. Our studies support the utility of B7-H4 as prognostic biomarkers in thyroid cancer patients undergoing cystectomy.

To date, the functional role of B7-H4 expression in tumor cells remains unclear and the mechanisms for B7-H4-mediated immune inhibition are not clear yet. It has been shown that the activation of the B7-H4 signaling pathway may lead to tumor cells escaping from immune surveillance (Flies and Chen, 2007; Arigami et al., 2011; Sun et al., 2012). Recent report showed that host B7-H4 may enable metastasizing cancer cells to escape local antitumor immune responses through interactions with the innate and adaptive immune systems (Abadi et al., 2013). Several evidences have demonstrated that the B7-H4 molecule promotes cancer progression via regulating T cell mediated antitumor immunity (Zang et al., 2003; Sica et al., 2003; Sun et al., 2012). Previous studies have showed that the higher expression of B7-family inhibitory molecules by tumor cells is significantly correlated with densities of TILs in human malignancies, such as gastric cancer and esophageal squamous cell carcinoma (Miyatake et al., 2007; Arigami et al., 2011; Chen et al., 2011; Zhang et al., 2013). We hypothesized that B7-H4 expression also involved in the thyroid cancer progression through regulating the T cell response. Tumor-infiltrating lymphocytes are one of the major immune components infiltrating solid tumors (Grabenbauer et al., 2006). In the present study, the B7-H4 expression status of primary tumor cells was inversely correlated with the number of infiltrating T lymphocytes in thyroid cancer. It is clear that further investigations are necessary to fully understand the mechanistic and functional role of B7-H4 on T cell mediated immune response and larger patient populations are need to determine whether B7-H4 expression has a role in predicting patient outcome in thyroid cancer.

In conclusions, our results demonstrate that B7-H4 is involved in thyroid cancer progression and tumor avoidance of immunosurveillance and could be a useful prognostic indicator for human thyroid cancer. Future studies on the biological behavior of thyroid cancer cells expressing B7-H4 may lead to a new immunotherapy blocking its signaling pathway in patients with thyroid cancer.

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