Expression levels of B7-H3 and TLT-2 in human oral squamous cell carcinoma

SHAN-SHAN ZHANG[1,2*], JING TANG[3*], SHU-YI YU[4], LI MA[5], FENG WANG[1], SHU-LE XIE[1,2], LONG JIN[1] and HONG-YU YANG[1]

[1]Department of Oral and Maxillofacial Surgery, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036; [2]Shantou University Medical College, Shantou, Guangdong 515000; [3]Department of Oral and Maxillofacial Surgery, Jingzhou Central Hospital, Jingzhou, Hubei 434000; [4]Department of Bioscience and Technology, Modern Analytical Center, Central South University, Changsha, Hunan 410000; [5]Discipline of Oral Rehabilitation, Faculty of Dentistry, The University of Hong Kong, Hong Kong, SAR 999077, P.R. China

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Abstract. The aim of the present study was to investigate the role of immune regulatory molecules B7-H3 [also known as cluster of differentiation 276] and triggering receptor expressed on myeloid cell-like transcript-2 (TLT-2) in patients with oral squamous cell carcinoma (OSCC). Human OSCC samples were obtained from 76 patients (female, 32; male, 44; age range, 23-81 years; median age, 50.9 years) that underwent resection for OSCC at Peking University Shenzhen Hospital (Shenzhen, China) between 2007 and 2010. In addition, control oral mucosal samples were obtained from 76 healthy individuals (female, 36; male, 40; age range, 21-62 years; median age, 45.3 years) during wisdom tooth extraction. Protein and gene expression levels of B7-H3 and TLT-2 were determined by immunohistochemical analysis and reverse transcription-polymerase chain reaction (RT-PCR). In the healthy oral mucosa samples, B7-H3 expression was identified to be weak, while the expression of TLT-2 was only detected sporadically in the cell membrane and cytoplasm. By contrast, the two regulatory molecules were widely expressed in the aforementioned localizations in human OSCC specimens upon immunohistochemical examination. Furthermore, quantitative RT-PCR confirmed the presence of significantly higher B7-H3 and TLT-2 expression levels in OSCC specimens compared with the oral mucosa of healthy individuals. The significantly higher expression levels of B7-H3 and TLT-2 in human OSCC specimens may indicate an inhibitory role of these molecules in the antitumoral immune response. To investigate interactions between these two molecules and individual antitumoral immune response in OSCC patients, prospective clinical studies with an adequate sample size are required.

Correspondence to: Professor Hong-Yu Yang, Department of Oral and Maxillofacial Surgery, Peking University Shenzhen Hospital, 1120 Lian Hua Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: hyyang192@hotmail.com

*Contributed equally

Abbreviations: OSCC, oral squamous cell carcinoma; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TLT-2, triggering receptor expressed on myeloid cell-like transcript-2

Key words: B7-H3/cluster of differentiation 276, triggering receptor expressed on myeloid cell-like transcript-2, tumor immunity, oral squamous cell carcinoma

Introduction

The antitumoral immune response is a complex physiological process involving a variety of immune cells and molecules, including membrane molecules and dissolubility factors (1). As well as the engagement of T cell receptors, costimulation with immune regulatory molecules is required for the optimal activation of T cells (2). The B7 family, a group of costimulatory and coinhibitory proteins, encompasses critical ligands that interact with known and unknown receptors on the surface of T lymphocytes, regulating stimulation or inhibition (3). The aberrant expression of members of the B7 family is considered to be a mechanism by which human malignancies may escape host immune surveillance (3-5).

B7-H3 [also known as cluster of differentiation (CD) 276], a member of the B7 family, is expressed on the surface of lymphoid cells, including dendritic cells, monocytes/macrophages and activated T cells. In addition, it is expressed on the surface of non-lymphoid tissue cells, including epithelial, anterior pituitary progenitor and muscle cells, as well as fibroblast-like synoviocytes (3,4). At present, the regulatory role of B7-H3 in tumor immunity remains controversial. A number of studies considered B7-H3 to be a costimulatory molecule promoting T-cell activation and proliferation and, thus, resulting in enhanced tumor immunity (4,5). However, other studies identified that a higher expression of B7-H3 in prostate, colon, ovarian, pancreatic and lung cancer tumors was positively correlated with more advanced tumor stages and poorer prognosis (6-12).

The immunoglobulin superfamily, triggering receptor expressed on myeloid cells (TREM), includes members such

as the TREM-like transcript-2 (TLT-2). TLT-2 is expressed on B cells, granulocytes and macrophages, and is constitutively expressed on $CD8^+$ T cells. Furthermore, TLT-2 expression is induced on $CD4^+$ T cells following their activation (11). Hashiguchi *et al* (13) proposed that TLT-2 may be a counter-receptor for B7-H3 and that the TLT-2/B7-H3 pathway costimulates $CD8^+$ T cell activation.

Oral squamous cell carcinoma (OSCC) presents a major health problem worldwide; a total of 274,300 new cases are diagnosed each year and the disease accounts for 128,000 mortalities, annually (14). OSCC is the most common type of cancer in the oral cavity, accounting for >90% of malignant neoplasms in this area (15). Approximately 96% of cases of OSCC are preceded by dysplasia, which presents as white epithelial lesions on the oral mucosa (leukoplakia). Dysplastic lesions in the form of erythroplakia (red lesions) are also commonly observed, which exhibit a 90% risk of malignant conversion (16). The majority of OSCC cases are diagnosed by oral examination and ~50% of patients diagnosed with OSCC succumb to the disease (17). At present, treatment modalities include surgery, radiotherapy and chemotherapy. However, despite the radical nature of treatment, recurrences are common (18). Thus, the identification of novel prognostic indicators is required to aid diagnosis and selection of the most effective treatment methods (19). To the best of our knowledge, thus far, no studies have been conducted investigating the roles of B7-H3 and TLT-2 in human OSCC. Therefore, the aim of the present study was to investigate and compare the gene and protein expression levels of B7-H3 and TLT-2 in human OSCC and healthy mucosal tissue samples.

Materials and methods

Patient selection. The present cross-sectional study was conducted in accordance with the principles outlined in the Declaration of Helsinki (20). Tissue specimens from patients and healthy subjects were collected according to the procedures approved by the Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China). Furthermore, all the patients and healthy individuals provided written informed consent prior to enrolment in this study.

Human OSCC samples were obtained from 76 patients (female, 32; male, 44; median age, 50.9 years; age range, 23-81 years) who had undergone tumor surgery for OSCC at Peking University Shenzhen Hospital between 2007 and 2010. All tumors were staged according to the the Union for International Cancer Control TNM classification system for head and neck tumors (21). A total of 46 samples exhibited high-grade histological differentiation, 26 cases exhibited moderate differentiation and 4 cases exhibited low-grade differentation. Furthermore, the study included 19 stage T1 tumors, 31 stage T2 tumors, 21 stage T3 tumors and 5 stage T4 tumors. The present study excluded patients with chronic disease, including diabetes, liver disease, tuberculosis or autoimmune diseases (such as rheumatoid arthritis or systemic lupus erythematosus), and patients that had previously undergone chemotherapy, radiotherapy or preoperative hormonal treatment. In addition, healthy oral mucosal samples were obtained from 76 control subjects (female, 36; male, 40; median age, 45.3 years; age range, 21-62 years) during wisdom tooth extraction.

Tissue sampling. Immediately after surgical resection, all the tissue samples were snap-frozen in liquid nitrogen (for RNA extraction) or fixed in 10% buffered formalin solution and stored at -80˚C, prior to embedding in paraffin (for histopathological and immunohistochemical analysis).

Immunohistochemistry. Immunohistochemical analyses were conducted using monoclonal goat anti-human B7-H3 (1:200; cat no. AF1027; R&D Systems, Inc., Minneapolis, MN, USA) and polyclonal goat anti-human TLT-2 (1:200; cat. no. 032737R; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies. Antigen retrieval was achieved by microwave pretreatment at 92˚C in 0.01 mol/l ethylene diamine tetraacetic acid buffer. Subsequently, xylene was used to deparaffinize the paraffin-embedded tissue sections (4-μm), followed by rehydration through graded alcohol into distilled water. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide in methanol for 10-15 min and the samples were blocked using an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) for 5 min. The OSCC sections were incubated with the primary antibodies overnight at 4˚C; however, the antibodies were omitted from the negative control samples. Next, a goat avidin-biotin complex staining kit (Santa Cruz Biotechnology, Inc.) was used to incubate the samples with a biotinylated rabbit anti-goat IgG secondary antibody (1:20; cat. no. BA-9001; Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China) and horseradish peroxidase enzyme-avidin conjugate, according to the manufacturer's instructions. The sections were subsequently counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). The B7-H3 and TLT-2 immunoreactivity of all samples was independently evaluated by two investigators in a blinded manner, based on the intensity of staining as follows: 0, no staining; 1+, weak diffuse cytoplasmic staining (<10% of the cancer cells may exhibit stronger intensity); 2+, moderate to strong granular cytoplasmic staining, present in 10-90% of the cancer cells; 3+, >90% of the tumor cells exhibited strong intensity. All specimens were evaluated by the two independent and blinded investigators using a multiheaded microscope (CX31; Olympus Corporation, Tokyo, Japan) and the consensus score was used for subsequent analyses (22).

Reverse transcription-polymerase chain reaction (RT-PCR). The frozen tissue was homogenized using a Mikro-Dismembrator U (Sartorius BBI Systems GmbH, Göttingen, Germany), according to the manufacturer's instructions. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized using the SuperScript® First-Strand Synthesis system (Invitrogen Life Technologies), according to the manufacturer's instructions. The sequences of the primers used for amplification were as follows: Forward, 5'-CCCATCCCACCCATAATTCTTACCC-3', and reverse, 5'-AGGCTCTGCCTTTTCTGCTGCTATG-3', for B7-H3 (product length, 182 bp; GenBank accession no., NM_025240.2); forward, 5'-CCCCTTTTACCACTGGTGTGATGGT-3', and reverse, 5'-GTGGTGCTGGTAGCAGTGAAGCTGT-3', for TLT-2 (product length, 118 bp; GenBank accession no., NM_024807.2); and forward, 5'-AAACTGGAACGGTGAAGGTG-3', and reverse, 5'-AGTGGGGTGGCTTTTAGGAT-3', for β-actin (product length, 166 bp;

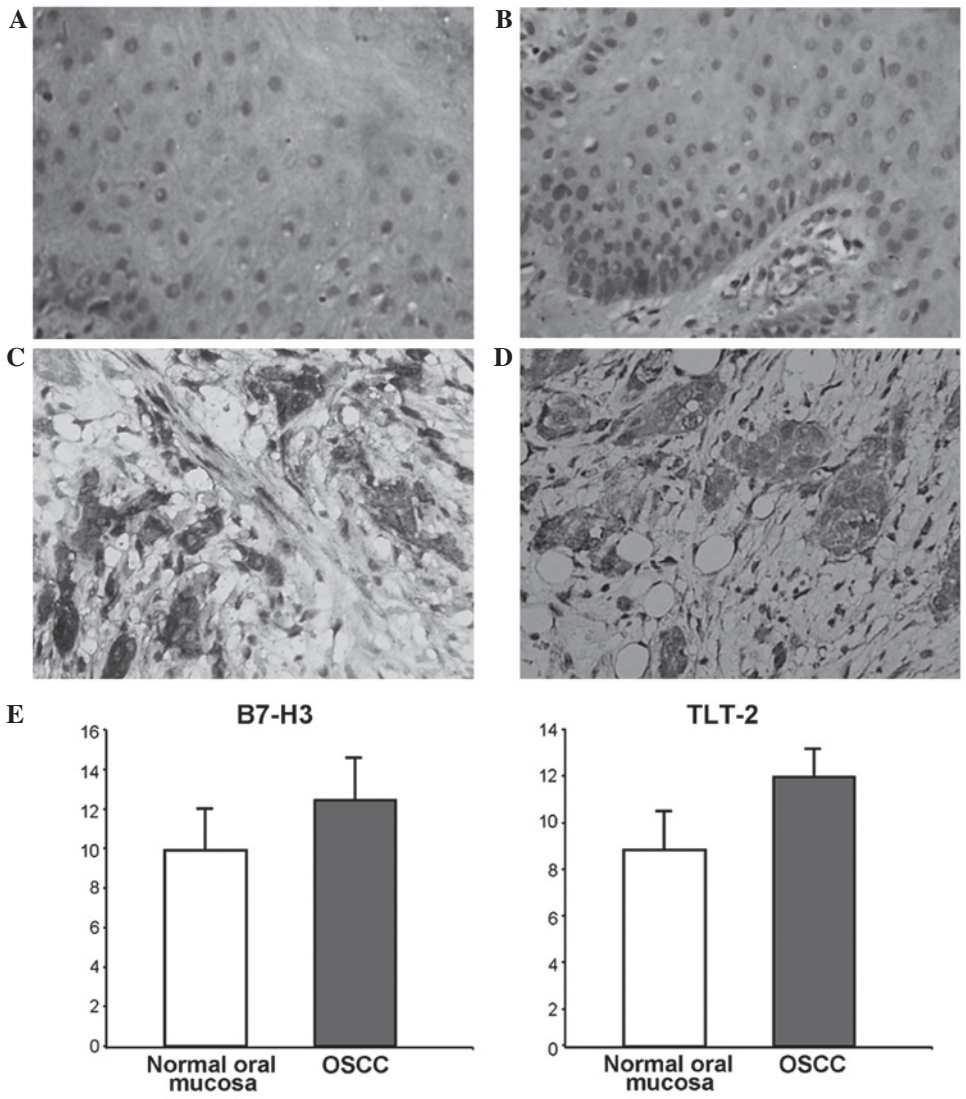

Figure 1. Immunohistochemical analysis demonstrating the expression levels of (A) B7-H3 and (B) TLT-2 in healthy oral mucosa samples; and the expression levels of (C) B7-H3 and (D) TLT-2 in OSCC samples (magnification, x400). (E) Quantitative reverse-transcription polymerase chain reaction analysis of B7-H3 and TLT-2 expression levels in OSCC and healthy oral mucosa specimens. *P<0.05 vs. normal oral mucosa tissues. TLT-2, triggering receptor expressed on myeloid cell-like transcript-2; OSCC, oral squamous cell carcinoma.

GenBank accession no., NM_001101.3). Quantitative RT-PCR (qRT-PCR) was conducted in a 20-μl reaction solution containing 2 μl cDNA, 15 μl 2X Power SYBR® Green PCR master mix (Applied Biosystems Life Technologies, Warrington, UK) and 200 nM of each pair of primers. The β-actin gene was used as an endogenous control. Amplification was performed for 38 cycles at 94˚C for 30 sec, 62˚C for 30 sec and 72˚C for 30 sec. The amplified PCR products were quantified by gel electrophoresis and visualized using the AlphaImager Mini System (ProteinSimple, San Jose, CA, USA). Each analysis was run in triplicate, the relative quantification of gene expression was determined using the comparative mean [standard deviation (SD)] and the results were normalized using the human β-actin housekeeping gene.

Statistical analysis. mRNA expression values between the OSCC and control groups were compared by performing two sample t-tests using SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA). Comparisons between B7-H3 and TLT-2 protein expression levels, and pathological features were evaluated by performing a χ^2 test and Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

Results

Immunohistochemical staining. Protein expression levels of B7-H3 and TLT-2 were detected in healthy oral mucosa and OSCC tissue samples. In the healthy oral mucosa, weak expression of B7-H3 (Fig. 1A) and TLT-2 (Fig. 1B) was sporadically detected within the cell membrane of epithelial cells. However, elevated expression levels of B7-H3 (Fig. 1C) and TLT-2 (Fig. 1D) protein were observed in the OSCC tissue samples. The two proteins demonstrated similar expression patterns, and were widely expressed within the cell membrane and cytoplasm of malignant epithelial, vascular endothelial and inflammatory cells.

Table I. Clinicopathological factors associated with B7-H3 and TLT-2 protein expression levels.

	B7-H3			TLT-2		
Factor	Normal	Increased	P-value	Normal	Increased	P-value
Tumor location			0.499[a]			0.371[a]
Tongue	14	24		16	22	
Gingiva	2	6		4	4	
Buccal mucosa	3	14		5	12	
Palate	2	2		1	3	
Floor of the mouth	4	5		1	8	
Tumor size			0.135[b]			0.212[b]
T1	10	9		9	10	
T2	10	21		12	19	
T3	4	17		6	15	
T4	1	4		0	5	
Nodal status			0.026[c]			0.021[c]
N0	17	18		18	17	
N1	6	26		8	24	
N2	2	7		1	8	
Metastatic status			0.073[d]			0.057[d]
M0	25	45		27	43	
M1	0	6		0	6	
Histological differentiation			0.011[e]			0.016[e]
High	21	25		22	24	
Moderate	4	22		5	21	
Low	0	4		0	4	

[a]P>0.05 vs. tongue. [b]P>0.05 vs. T1. [c]P<0.05 vs. N0. [d]P>0.05 vs. M0. [e]P<0.05 vs. high. Tumor Node Metastasis classification of malignant tumors was used to classify the tumor size, nodal status and metastasis status. TLT-2, triggering receptor expressed on myeloid cell-like transcript-2.

RT-PCR. The mRNA expression levels of B7-H3 and TLT-2 were detected in the OSCC and healthy oral mucosal tissue samples. As expected, the RT-PCR products of B7-H3 and TLT-2 were 182 and 118 bp long, respectively.

qRT-PCR. The mRNA expression levels of B7-H3 and TLT-2 were detected in all the OSCC and healthy oral mucosa specimens. The expression levels of B7-H3 mRNA in the OSCC and healthy oral mucosa tissues were 12.449 (SD, 2.14) and 9.899 (SD, 2.12), respectively. By contrast, the expression levels of TLT-2 mRNA in the OSCC and healthy oral mucosa tissues were 11.567 (SD, 1.24) and 8.493 (SD, 1.67), respectively. Compared with the expression in healthy oral mucosa samples, the expression levels of B7-H3 (P=0.0004) and TLT-2 (P<0.0001) were significantly higher in OSCC specimens (Fig. 1E).

Clinicopathological factors associated with B7-H3 and TLT-2 protein expression. The statistical correlation between the B7-H3 and TLT-2 protein status and specific clinicopathological parameters was investigated using χ^2 analysis, as indicated in Table I. B7-H3 and TLT-2 overexpression were not associated with the location, size or metastatic status of the tumor. However, the B7-H3 and TLT-2 expression levels were positively associated with lymph node metastasis (P=0.026 and P=0.021, respectively) and negatively correlated with histological differentiation (P=0.011 and P=0.016, respectively).

Discussion

The optimal activation of antigen-specific lymphocytes requires a combination of T-cell receptor and costimulatory signals; however, this activation may be inhibited by coinhibitory signals (3-5). Recent studies demonstrated that specific B7 family ligands, including B7-H1, B7-DC, B7-H3 and B7-H4, were highly expressed in a wide spectrum of human cancer types, including esophageal, gastric, lung, ovarian, breast, renal and pancreatic cancer. Their expression levels positively correlated with a more advanced tumor stage and poor prognosis (23-30). Furthermore, it was detected that B7 molecules contribute to an immune-suppressive tumor microenvironment (31). However, the role of B7-H3 in adaptive immune responses remains controversial. B7-H3 was initially identified as a costimulatory molecule that engages with its receptor on T cells to promote T-cell activation and the secretion of interferon-γ (13,32). However, a number of recent studies have demonstrated that B7-H3 exhibits an inhibitory effect during autoimmunity by coinhibiting T-cell function (6,33).

OSCC is the most common type of cancer in the oral cavity; however, the role of the B7 family in OSCC has yet to be fully investigated. Tsushima *et al* (34) examined the expression of five B7 molecules, including B7-H1, B7-DC, B7 h, CD80 and CD86, in nine human OSCC cell lines and four biopsied OSCC specimens. Various levels of B7-H1 and B7-DC expression were detected in the majority of the investigated OSCC cell lines; however, B7-H1 was detected in all the cell lines, as well as the biopsied human OSCC specimens. To the best of our knowledge, the present study investigated for the first time the protein and gene expression levels of B7-H3 in human OSCC. The results indicated that B7-H3 was overexpressed in OSCC and was a critical determinant for predicting lymph node metastasis and histological differentiation. Furthermore, the results revealed an inhibitory role of B7-H3 in tumor immunity against OSCC.

The interaction between B7-H3 and TLT-2 remains controversial. Hashiguchi *et al* (13) have previously proposed TLT-2 as a counter-receptor to B7-H3. Furthermore, the same authors considered that the TLT-2/B7-H3 pathway costimulates T cell activation; however, alternative studies identified no evidence of an interaction between B7-H3 and TLT-2 (35). The data presented in the current study demonstrated a similar expression pattern of TLT-2 and B7-H3 in OSCC and healthy oral mucosa specimens. The distribution of B7-H3 and TLT-2 overexpression in OSCC indicated a correlation with inflammation, although the mechanism of the association between inflammation and the overexpression of B7-H3 or TLT-2 requires further investigation. In addition, the present data strongly indicated that TLT-2 and B7-H3 are critical determinants in the prediction of lymph node metastasis in OSCC tumors. However, the underlying mechanism through which these two molecules contribute to lymph node metastasis in oral cancer requires further investigation.

In conclusion, the present study identified B7-H3 and TLT-2 overexpression in OSCC specimens. Furthermore, the B7-H3 and TLT-2 expression levels were significantly associated with lymph node metastasis and histological differentiation in the OSCC samples. Therefore, B7-H3 along with TLT-2 may exhibit an inhibitory role on the antitumoral immune response in OSCC. In order to investigate the interactions between these two molecules and individual antitumoral immune responses in OSCC patients, prospective clinical studies with adequate sample sizes should be conducted.

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