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ORIGINAL ARTICLE

Immunohistochemical Study of PD-1/PD-L1 Axis Expression in Oral Tongue Squamous Cell Carcinomas: Effect of Neoadjuvant Chemotherapy on Local Recurrence

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Abstract

While neoadjuvant chemotherapy (NAC) for patients with oral tongue squamous cell carcinoma (OTSCC) may improve tumor microenvironment, it may lead to local immune suppression caused by residual cancer cells. The efficacy of NAC is therefore controversial. In our study, we investigated tumor microenvironments after NAC using immune checkpoint molecules, and evaluated the association between tumor microenvironments, clinicopathological factors and outcomes. We reviewed the records of 121 patients who underwent radical surgery for OTSCC between April 2001 and March 2015. Patients with a positive surgical margin and a follow up period of less than 6 months were excluded. For these patients, programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) expressions were immunohistochemically examined. The expression of PD-1 and PD-L1 were significantly associated with local recurrence in patients with OTSCC ($P < 0.01$ and $P < 0.01$, respectively). We found a significant decrease in 5-year disease specific survival rate for patients with combined PD-1+/PD-L1+ expressions ($P < 0.05$). In the subgroup analysis of local recurrence between the NAC treated group and those who received surgery alone, high levels of PD-1 and PD-L1 expressions were significantly found in the former, but not in the latter group. Local recurrence in the NAC-treated group may contribute to local immune suppression in OTSCC. NAC lead to local immune suppression and immune checkpoint molecules play an important role in local recurrence in patients with OTSCC who received NAC. NAC modality can't be recommended for patients with OTSCC at present.

Keywords PD-1 · PD-L1 · Local recurrence · NAC

Introduction

Oral squamous cell carcinoma (OSCC) represents approximately 2% of all human cancers and has a predilection for the tongue [1, 2]. The 5-year survival of OSCC is generally over 50%, while that of oral tongue squamous cell carcinoma (OTSCC) is less than 50%. Additionally, OTSCC is associated with a poor prognosis [2, 3]. The current standard treatment of OSCC for most patients is surgery, while post-operative concurrent chemo/radiotherapy is indicated for patients with high-risk OSCC [4]. Meanwhile, several clinical studies have demonstrated that neoadjuvant chemotherapy (NAC) would be beneficial in reducing distant metastasis and to preserve organ function [5, 6]. However, there is no significant clinical evidence that NAC improves survival and so its efficacy is controversial.

The immune response against cancer cells mediated by CD8- positive cytotoxic T cells play an important role in local tumor control and disease-free survival. However, exhausted T cells promoted by tumor-bearing hosts fail to proliferate and exert effector functions such as cytotoxicity and cytokine secretion in response to antigen stimulation [7]. One of the most promising pathways for understanding this hypothesis is the interaction between programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1). In tumor microenvironments, expression of PD-1 is thought to be both a marker for and contributor to the exhaustion on the surface of several

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immune cells, particularly cytotoxic T-cells and increases the susceptibility of these cells to PD-L1 mediated death signals in those with head and neck squamous cell carcinoma SCC [7, 8]. Other than tumor-bearing hosts, immune suppressor cells such as myeloid-derived suppressor cells, T-regulatory cells and tumor associated macrophages lead to the expression of PD-L1 and the secretion of immunosuppressive cytokines [9].

Regarding tumor microenvironments after NAC, we previously reported that residual cancer cells (i.e. NAC surviving cells) exhibiting the characteristics of cancer stem cell (CSC) are involved in the mode of local recurrence [10]. Peng et al. have reported that chemotherapy improves local immune state, affecting the response of the tumor to treatment but can also lead to immune suppression caused by residual cancer cells through PD-L1 up-regulation [11]. Therefore, in addition to our previous report [10], residual cancer cells induce local immunosuppression and may also be involved in the mode of local recurrence. However, it is unclear whether expression of PD-1 or PD-L1 is clinically useful in the microenvironment after NAC. We therefore investigated the relationship between NAC and PD-1/PD-L1 expression and evaluated this association with clinicopathological factors and outcomes of patients with OTSCC.

Materials and Methods

Patients

Ethical approval was obtained from the independent ethics committee of our hospital (Approval no. 15061128). We retrospectively reviewed the records of 121 patients who underwent radical surgery for OTSCC between April 2001 and March 2015. Patients with a positive surgical margin and a follow up period of less than 6 months were excluded. In this study, extensive pretreatment evaluations, including physical examinations, computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography (US) and/or positron emission tomography/computed tomography (PET/CT) were performed in all patients. The tumor stage was classified according to the TNM classification of the International Union Against Cancer [12].

Treatment and Pathologic Examination

In our institute, treatment of OTSCC for most patients is surgery, and postoperative concurrent chemo/radiotherapy was applied for patients with high-risk factors according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guideline [4]. However, NAC was selected when patients hesitated to undergo surgery, or when surgery was not possible because of a busy schedule in the past. The regimen of NAC consisted of a platinum-based chemotherapy. Modifications to chemotherapy dosages were made using standard criteria to prevent toxicity. The resection after NAC was underwent in old margin. Elective neck dissection was not performed routinely in our institution.

Tumor histologic differentiation was defined according to the WHO classification. Pattern of invasion (POI) was classified according to Bryne's classification [13]. The depth of invasion (DOI) was measured from the surface of the adjacent mucosa to the most invasive front of the tumor and was subsequently classified as either ≥4 or < 4 mm. NAC histological response was evaluated according to the following criteria of the Japan Society of Oral Tumors: Grade 0, ineffective; Grade 1a, very slightly effective; Grade 1b, slightly effective, Grade 2, moderately effective; and Grade 3, markedly effective. The NAC group was divided into a non-responder group (Grade 0 and 1a) and responder group (Grade 1b, 2 and 3) [14].

Immunohistochemical Staining and Evaluation

Sections were deparaffinized in xylene followed by ethanol, and then washed with H_2O. For antigen retrieval, the sections were boiled in 10 mM citrate buffer (pH 6.0) for 5 min at 121 °C using an autoclave. After endogenous peroxidases were blocked by incubation with 0.3% H_2O_2 in methanol for 30 min, tissue sections were washed in PBS followed by incubation with the primary antibodies at 4 °C overnight. Negative controls were incubated with phosphate-buffered saline (PBS) alone. The following rabbit polyclonal primary antibodies from Abcam (Cambridge, UK) were used: PD-1 (ab137132; dilution 1:200) and PD-L1 (ab156361; dilution 1:250). Immunohistochemical staining was performed using the EnVision method (EnVision+ kit; Dako, Glostrup, Denmark). After incubation with the secondary antibody, the sections were washed in PBS and immersed in diaminobenzidine solution. The samples were counterstained with Meyer's hematoxylin and mounted.

The expression of PD-1 was evaluated by counting PD-1-positive tumor infiltrating lymphocytes (TILs) at the invasion front of the tumor tissue in each of the two visual fields; specimens displaying more than 10 PD-1 expressing TILs were defined as positive, because the mean numbers of PD-1 expressing TILs were approximately 10–15 [15]. The expression of PD-L1 proteins was evaluated by counting the presence of specific staining in the cytoplasm and nuclei of tumor cells at the invasion front of the tumor in each of two visual fields. The cut-off value was defined as the point when more than 5% of tumor cells stained positive [15, 16]. The intensity score was also evaluated as a reference value (0, no staining; 1, weak; 2, moderate; 3, strong). The cut-off value was defined as the point when more than 'weak' of tumor cells stained positive. All immunohistochemical assessments

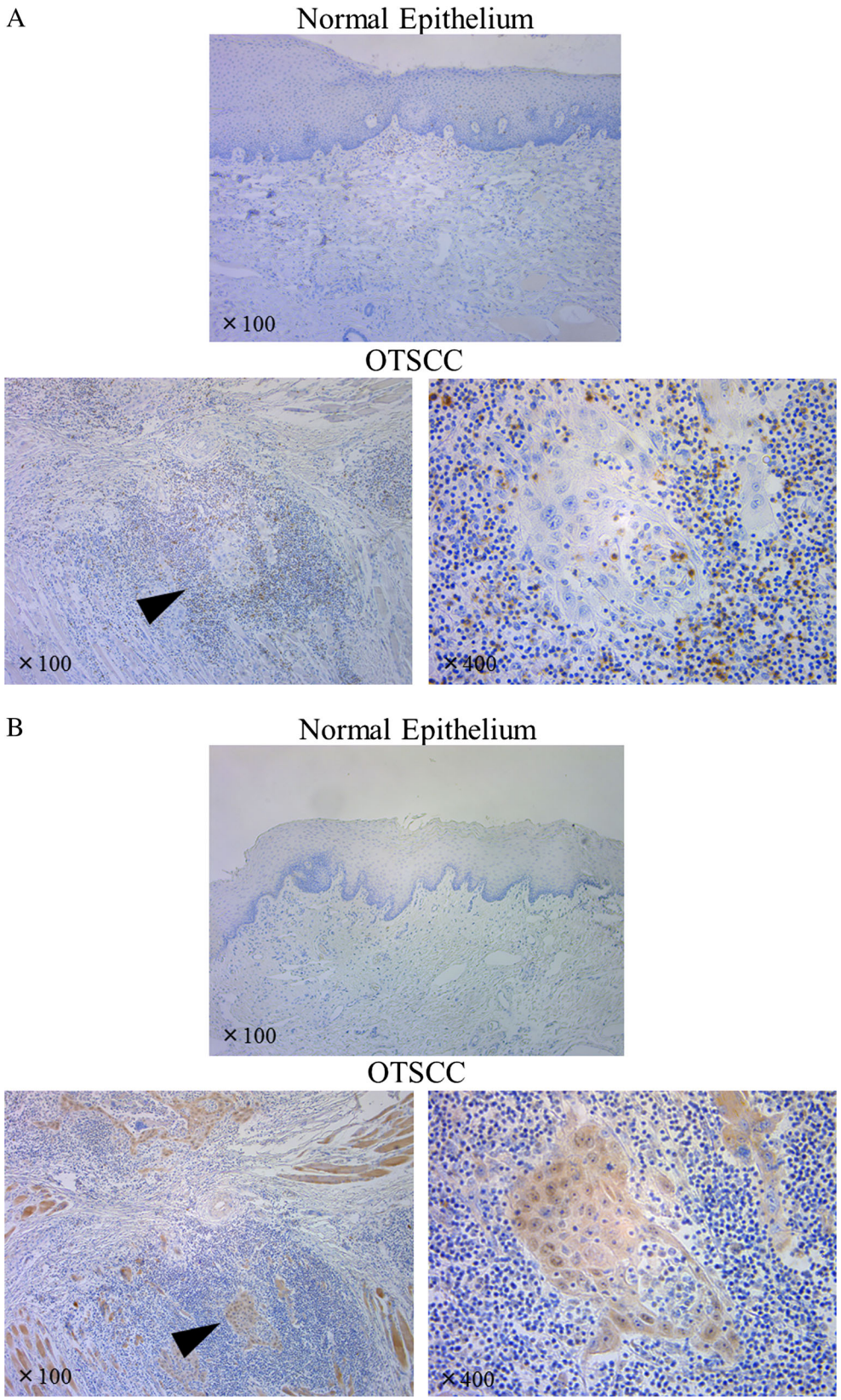

Fig. 1 Representative immunohistochemical staining for PD-1 and PD-L1. **a** Negative staining for PD-1 is seen in normal subepithelium. OSCC demonstrates strong PD-1 was expressed primarily in the lymphocyte at the infiltrating and/or surrounding tumor nests (original magnification ×100 and ×400 for the lower left and right panels, respectively). **b** Negative staining for PD-L1 is seen in the normal epithelium. OSCC demonstrates strong PD-L1 cytoplasmic and nuclear expression (staining index of 12, original magnification ×100 and ×400 for the lower left and right panels, respectively)

were performed by two examiners in a blinded fashion. Specimens of normal oral mucosa from 10 healthy individuals were used as controls.

Statistical Analyses

Associations between the expression of targeted molecules and clinicopathological features were analyzed by Fisher's exact test. Continuous data are presented as means ± standard deviation. Survival analyses were calculated by the Kaplan-Meier method and compared by the log-rank test. Differences between the two groups were compared by the *t*-test. *P* values of less than 0.05 were considered significant.

Results

Expression of PD-1 and PD-L1 in OTSCC

Among the 121 patients with OSCC, 54.5% were positive for PD-1 and 57.9% were positive for PD-L1 immunohistochemically. PD-1 was expressed primarily in the lymphocytes at the infiltrating and/or surrounding tumor nests. PD-L1 was expressed primarily in the cytoplasm and nuclei of tumor cells, with particularly strong expression observed at the invasive front. In the normal oral epithelium, expressions of these molecular markers were absent (Fig. 1a, b).

Association of PD-1 and PD-L1 Expression with Clinicopathological Factors and Survival

To investigate the clinical significance of the PD-1 and PD-L1 expression levels in OTSCC, the association was examined between expression levels of these molecules and clinicopathological factors. The expression of PD-1 was significantly associated with local recurrence and regional recurrence ($P < 0.05$). The expression of PD-L1 was significantly associated with N classification, DOI, PNI (perineural invasion) and local recurrence ($P < 0.05$) (Table 1).

The 5-year disease-specific survival (DSS) rates according to PD-1 and PD-L1 expression were determined. Univariate analyses by the log-rank test and the Kaplan-Meier method showed that PD-L1 was significantly associated with the 5-year DSS ($P < 0.05$) and decreased 5-year DSS in patients with PD-L1+ expression (Fig. 2a, b). Moreover, there was a significant correlation between combined PD-1+/PD-L1+ expression and combined PD-1-/PD-L1- expression ($P < 0.05$) (Fig. 2c). Overall, the immunohistochemical analyses showed that a high expression of both PD-1 and PD-L1 were significantly associated with local recurrence and decreased 5-year DSS in patients with combined PD-1+/PD-L1+ expression.

Table 1 Association of PD-1 and PD-L1 expressions with clinicopathological factors

		n	PD-1		*P* value	PD-L1		*P* value
			–	+		–	+	
Histopathology	Normal epithelium	10	10	0	<0.001	10	0	<0.001
	OTSCC	121	56	65		41	70	
Sex	Male	65	30	35	1.000	27	38	1.000
	Female	56	26	30		24	32	
Age	64 ≥	63	30	33	0.855	28	35	0.712
	64 <	58	26	32		23	35	
T classification	T1 + T2	109	50	59	1.000	45	64	0.759
	T3 + T4	12	6	6		6	6	
N classification	N0	102	49	53	0.456	47	55	0.047
	N1 + N2 + N3	19	7	12		4	15	
Differentiation	well	114	52	62	0.703	48	66	1.000
	moderate. Poor.	7	4	3		3	4	
Pattern of invasion	Grades 1/2	95	44	51	1.000	43	52	0.262
	Grades 3/4	26	12	14		8	18	
Depth of invasion	4 mm ≥	54	29	25	0.149	33	21	<0.001
	4 mm <	67	27	40		18	49	
Perineural invasion	No	84	42	42	0.24	41	43	0.029
	Yes	37	14	23		10	27	
Treatment	Surgery alone	101	44	57	0.462	40	61	0.224
	NAC	20	11	9		11	9	
Local recurrence	No	104	53	51	0.009	49	55	0.004
	Yes	17	3	14		2	15	
Regional recurrence	No	96	49	47	0.045	44	52	0.119
	Yes	25	7	18		7	18	

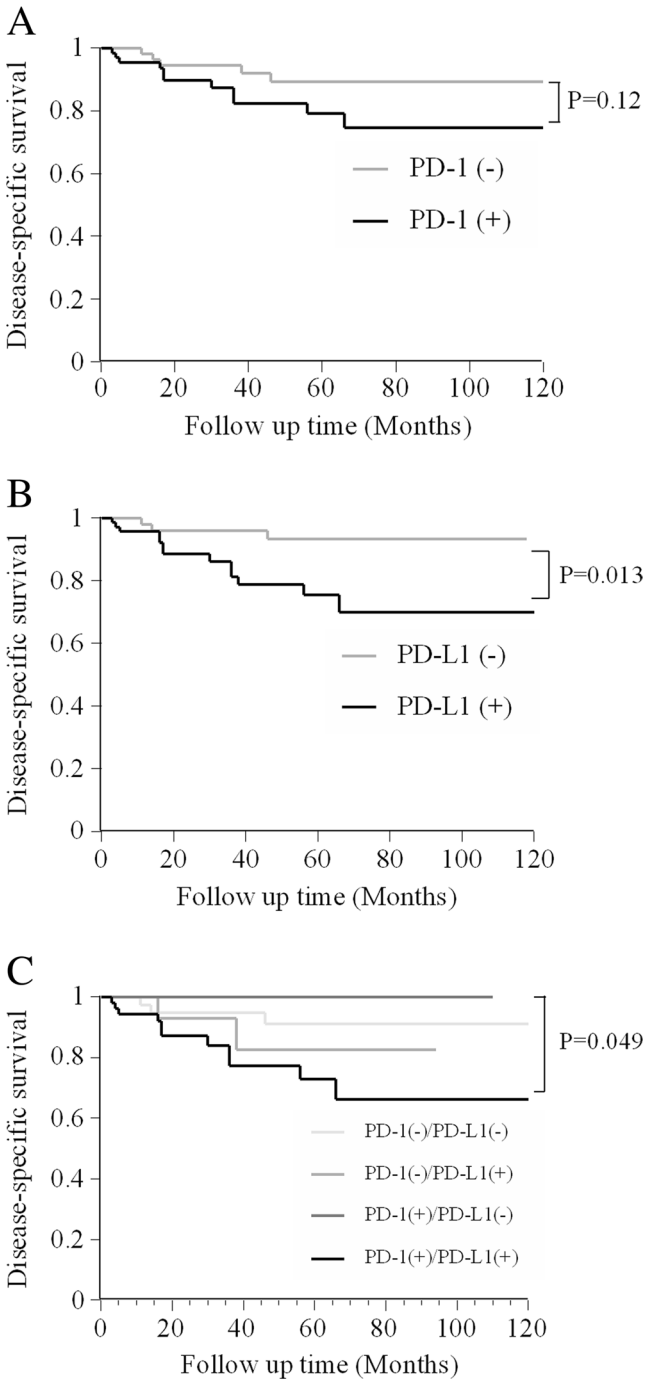

Fig. 2 Kaplan-Meier curve of the 5-year disease-specific survival (DSS) rate. No significant correlation between (**a**) PD-1-positive and -negative patients was observed, (**b**) but significant correlation between PD-L1-positive and negative-patients was observed in the 5-year DSS rate ($P = 0.12$ and 0.013 for PD-1 and PD-L1, respectively). (**c**) Significant correlation between combined PD-1+/PD-L1+ cases and combined PD-1-/PD-L1- cases ($P < 0.05$)

Association of NAC with PD-1 and PD-L1 Expression

There was a significant correlation between local recurrence and immune checkpoint molecules (PD-1 and PD-L1) in all patients. Therefore, to examine the effect of NAC on local tumor microenvironments, a subgroup analysis was performed on two different groups: those who had surgery alone and those who had NAC plus surgery. There was a significant correlation between local recurrence and PD-1 or PD-L1 positivity in the NAC-treated group but not in those who had surgery alone (Tables 2 and 3). No significant correlation between the histological tumor response after NAC and immune checkpoint molecular expression was observed. No marked difference in positivity rates of PD-1 and PD-L1 was observed between those who had surgery alone (55.4%, 60.4%, respectively) and those had NAC plus surgery (45.0%, 45.0%, respectively). However, a very high expression of immune checkpoint molecules were observed in NAC treated patients with local recurrence compared with those treated with surgery alone who had local recurrence (Fig. 3a, b), and the prognosis of these patients was poor. In contrast, immunopositivity in NAC treated patients without local recurrence revealed low intensity staining (Fig. 3c, d).

Discussion

Local recurrence in patients with OTSCC is a high potential risk factor for survival [10, 17]. Although local immune suppression caused by NAC may contribute to local recurrence, it is unclear how high PD-1/PD-L1 axis expression is associated with local recurrence and survival in patients with OTSCC. We therefore focused on the expressions of immune checkpoint molecules in the most invasive front. Our study hypothesized that high PD-1/PD-L1 axis expression is associated with local recurrence after NAC and also associated with poor survival.

As clinicopathological evidence supporting our hypothesis, we found that the expression of PD-L1 was associated with N classification, DOI, PNI and local recurrence ($P < 0.05$, respectively), and the expression of PD-1 was associated with local recurrence ($P < 0.05$). We also found that high expression levels of the PD-1/PD-L1 axis were associated with decreased 5-year DSS. These results suggest that the PD-1/PD-L1 axis affects the progression of OSCC. To the best of our knowledge, previous retrospective studies that analyzed the expression of PD-L1 and/or PD-1 axis immunohistochemically suggested that these molecules were associated with a high degree of malignancy and reduced survival in patients with head and neck cancers [18–22]. Our results are in accordance with those reports. On the other hand, Hanna et al. reported that high PD-L1 expression is associated with improved survival and lower recurrence risk in young women with OSCC [23]. It was speculated that the reason for this good outcome was due to increased tumor PD-L1 expression which may reflect a prior infiltrative CD8 positive T cell response that can delay time to recurrence or help prevent it. However, more recently, it was reported that not only protein expression but also mRNA

Table 2 Relationship between PD-1, PD-L1 and local recurrence by subgroup (surgery alone and NAC) assay

Factors	NAC-treated group			*P* value	Surgery alone group			*P* value
	n	Local recurrence ($n = 20$)			n	Local recurrence ($n = 101$)		
		–	+			–	+	
PD-1								
negative	11	11	0	0.026	45	42	3	0.136
positive	9	5	4		56	46	10	
PD-L1								
negative	11	11	0	0.026	38	38	2	0.071
positive	9	5	4		50	50	11	

Table 3 Correlation of the histopathological response after NAC with local recurrence or PD-1 and PD-L1 expressions

Factors	n	histopathological response		*P* value
		non-response	response	
PD-1				
negative	11	7	4	1.000
positive	9	5	4	
PD-L1				
negative	11	7	4	1.000
positive	9	5	4	

expression in circulating tumor cells isolated in the peripheral blood is associated with poor survival [24].

With regard to local recurrence, we performed a subgroup analysis of two groups: those who had surgery alone and those who had NAC plus surgery. We found that a high expression of PD-1 and/or PD-L1 in the NAC treated group was significantly associated with local recurrence ($P < 0.05$, respectively). In regard to local immunogenic change associated with chemotherapeutic treatments, Ock et al. reported that PD-L1 expression in approximately 70% of patients with head and neck SCC is up-regulated after cisplatin containing chemotherapy [25]. In this report, the underlying mechanism of expression of PD-L1 was change in response to exogenous signals. Shalapour et al. reported that IgA^+ plasmocytes that depend on TGF-β signaling after treatment with oxaliplatin induce CD8-positive cell exhaustion and suppress anti-tumor cytotoxic T cell responses through PD-L1 and IL-10 expression [26]. In this report, local immune

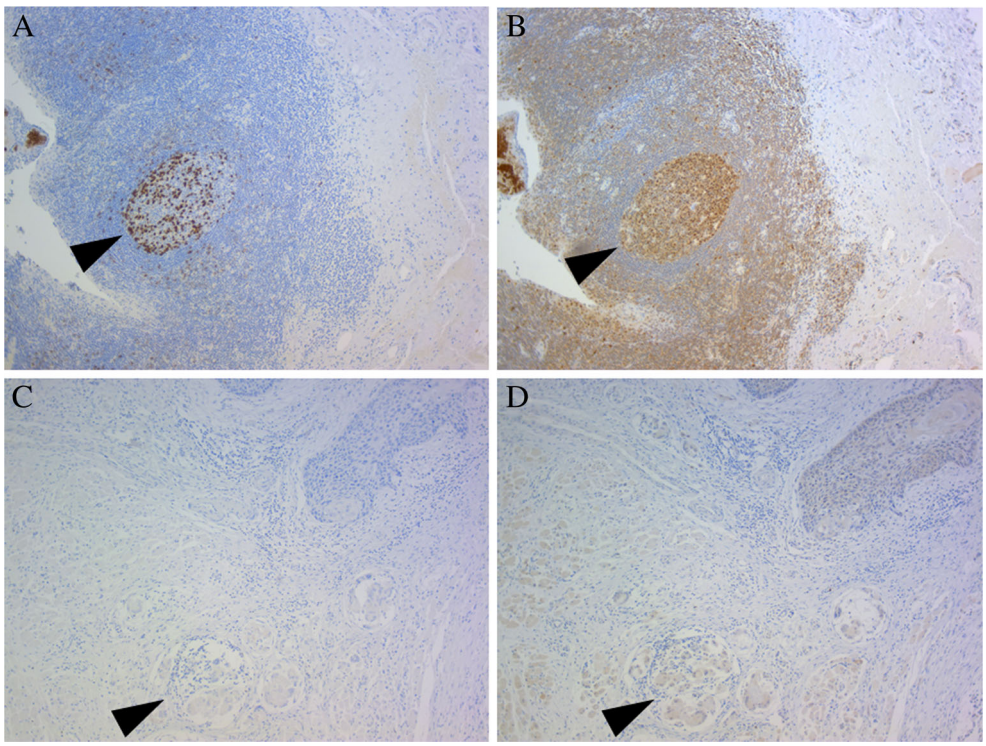

Fig. 3 Representative immunohistochemical staining for PD-1 and PD-L1 in NAC-treated group. Immunopositivity of PD-1 (**a**) and PD-L1 (**b**) showed significantly high expression in NAC- treated group with local recurrence compared with surgery alone group, whereas immunopositivity of PD-1 (**c**) and PD-L1 (D) in NAC-treated group without local recurrence was faint

suppressor cells were induced through inflammatory cytokines after cancer cell death occurred by chemotherapy. Moreover, Peng et al. have reported that paclitaxel containing chemotherapy lead to immune suppression caused by residual cancer cells through PD-L1 upregulation [11]. We previously proposed that as the most invasive front became more difficult to identify after modification by NAC, undetected residual cancer cells after NAC may have presented in the surgical margins [10]. Most residual cancer cells will be detected in tumor tissue at the resection margin when the resection is incomplete. However, the number of residual cancer cells is too small to be detected by routine histopathology [27]. Taken together, one of the mechanisms of local recurrence behind the NAC treated group in our study suggested that chemotherapy lead to immune suppression caused by inflammatory cytokines and/or undetected residual cancer cells present in the surgical margin. Hirakawa et al. reported that patients who had effective histological tumor responses to NAC had better loco-regional control and survival than those who had ineffective responses to NAC [14]. However, in our study, the relationship between the histological tumor response and immune checkpoint molecular expression was unclear.

A potential weakness of this immunohistochemical study is its retrospective nature and the small patient cohort from a single institution. Moreover, there was some inherent bias in conducting our study since the data was recorded by the authors. Although the patient characteristics and treatment strategy in OTSCC, including chemotherapeutic agents, were unified to reduce the bias associated with the retrospective study design, the statistical power of our study may be low. Therefore, a multicenter study with an increased number of patients is needed.

In conclusion, NAC lead to local immune suppression and immune checkpoint molecules play an important role in local recurrence in patients with OTSCC who received NAC compared with patients who received surgery alone. Therefore, we cannot yet recommend the NAC modality for patients with OTSCC at present. Recently, nivolmab, an anti- PD-1 antibody, was approved March 2017 and patients with recurrent HNSCC who had disease progression after platinum-based chemotherapy vhave been treated effectively [28]. Our study suggests that inhibition of the PD-1/PD-L1 axis may be a good strategy to improve prognosis in OTSCC patients with local recurrence after NAC.

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Compliance with Ethical Standards

Conflict of Interest None declared.

Ethical Approval All procedures of this study involving human participants were in accordance with the the 1964 Helsinki declaration.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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