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Original Article

The Immunological Impact of Chemotherapy on the Tumor Microenvironment of Oral Squamous Cell Carcinoma

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Anticancer drugs induce cell-cycle arrest and apoptosis not only in tumor cells, but also in immune cells. However, many preclinical and clinical findings show that some chemotherapeutic agents can improve the anti-tumor efficacy of immunotherapy. We immunohistochemically analyzed the degree of immune cell infiltration and the relevance of programmed cell death 1 ligand-1 (PD-L1) expression in surgically resected oral squamous cell carcinoma (OSCC) specimens from patients who had undergone pretreatment with certain chemotherapies and other patients without pretreatment. We divided the patients into the group of neoadjuvant chemotherapy (NAC) patients (n = 8) and the nNAC (without NAC) patient group (n = 10). We observed that NAC induced infiltrations of CD4, CD8 T cells and CD56 NK cells into the tumor microenvironment. Decreased numbers of Tregs and PD-1-positive cells were observed in the NAC group. No significant difference was observed in the degree of immune-cell infiltration between the patient groups except for CD56 NK cells in the stroma and PD-1 cells in cancer nests. Eighty percent of the nNAC specimens showed intermediate-to-strong PD-L1 protein expression, whereas 75% of the NAC specimens showed down-regulation of the PD-L1 protein, indicating the effectiveness of the chemotherapeutic treatment before surgery.

Key words: oral squamous cell carcinoma, programmed cell death 1 ligand-1, tumor microenvironment, neoadjuvant chemotherapy, immunohistochemistry

Oral squamous cell carcinoma (OSCC) is one of the top 10 most common malignancies worldwide [1]. Despite some progress in diagnostics and therapeutic options, the 5-year overall survival rate for OSCC patients has stagnated at 40-50% over the last 40 years [2-5]. The low survival rates in combination with the significant toxicities caused by the current treatment strategies used for OSCC emphasize the need for new treatment options. In OSCC and other cancers, the immune system plays an important role in the development and progression, and recent cancer immunotherapies have focused on immunoregulatory molecules in the tumor microenvironment, including immune checkpoints [6].

For example, programmed cell death-1 (PD-1) is expressed in activated T and B cells. Its major ligand (PD-L1) is expressed in antigen presenting cells, as well as in tumor cells. Aberrant PD-L1 expression in cancers facilitates an escape from immune attack [7,8]. Nivolumab, an anti-PD-1 therapeutic agent, was recently approved by the U.S. Food and Drug

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Administration for the treatment of various malignancies, and it is currently being investigated in clinical phase III trials for the treatment of OSCC [9-12].

The tumor microenvironment is known to influence the curative and convalescent effects in patients who have undergone chemotherapeutic treatment, due to the association between the tumor microenvironment and immune checkpoint inhibitors [13-19]. In the present study, we retrospectively analyzed the degree of immune cell infiltration and the relevance of PD-L1 expression in surgically resected OSCC specimens from patients with or without pretreatment with certain chemotherapies.

Materials and Methods

OSCC patients. Eighteen patients were evaluated in this study. Their characteristics are summarized in Table 1. We retrospectively divided the patients into 2 groups: the NAC group (n=8) of patients who received neoadjuvant chemotherapy of FP (3 patients), CDDP (1 patient), or S-1 (4 patients) followed by surgical resection; and the nNAC group (n=10) of patients who underwent surgical resection without any neoadjuvant chemotherapy. The median interval between the end of chemotherapy and the surgical resection for 8 patients in the NAC group was 19.6 days. The mean age at diagnosis was 71 years (54-85 years) in the NAC group and 65 years (32-81 years) in the nNAC group. In the NAC group, there were 3 stage II patients, 1 stage III patient, 3 stage IVA patients, and 1 stage IVB patient. In the nNAC group, there were 4 stage I patients, 2 stage II patients, 2 stage III patients, and 2 stage IVA patients.

The white blood cell (WBC) counts before the surgical resection in each patient are also shown in Table 1. Cancer tissue specimens were obtained surgically from all of the patients at the Department of Maxillofacial Surgery at Okayama University Hospital between 2004 and 2007. Blood samples were also obtained from each patient. Written informed consent for the analyses in the present study was obtained from all patients. The study was conducted in accord with the Declaration of Helsinki regarding medical protocol and ethics. The Ethics Committees of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital approved this study (Approval No. 1608-031).

Table 1 Patient characteristics

	nNAC (n=10)	NAC (n=8)	
Age (years)	65 (32–81)	71 (54–85)	n.s.
Sex (male/female)	4/6	3/5	n.s.
Clinical Stage I/II/III/IVA/IVB	4/2/2/2/0	0/3/1/3/1	n.s.
Preoperative WBC (range) ($\times 10^3/\mu$L)	5.61 (3.0–9.3)	4.60 (2.7–6.9)	n.s.

n.s.: no significant.

Immunohistochemistry. The tumor specimens were fixed with buffered formalin and embedded in paraffin. Sections (5 μm) were placed on glass slides, heated at 60°C for 20 min, and then deparaffinized. The sections were treated by autoclaving for 5 min in 0.01M citrate buffer (pH 6.0) for CD56, HLA class I, CCDC62-2, and MAGE-A antigen retrieval.

For CD4, CD8, FOXP3, CCR4, PD-1, and PD-L1 antigen retrieval, slides in 0.01M citrate buffer (pH 9.0) were heated in a microwave for 5 min. Endogenous peroxidase was then blocked by treatment with 0.3% H_2O_2 in methanol for 30 min at room temperature. After the blocking of endogenous peroxidase, the sections were incubated overnight at 4°C with mouse monoclonal anti-CD4 antibody (1F6, Leica Biosystems, Nussloch, Germany), mouse monoclonal anti-CD8 antibody (C8/144B, Dako, Glostrup, Denmark), mouse monoclonal anti-CD56 antibody (1B6, Leica Biosystems), mouse monoclonal anti-CCR4 antibody (POTELIGEO®TEST, Kyowa Medex, Tokyo, Japan), mouse monoclonal anti-FOXP3 antibody (236A/E7, Abcam, Cambridge, UK), mouse monoclonal anti-PD-1 antibody (NAT105, Abcam), mouse monoclonal anti-PD-L1 antibody (27A2, MBL, Nagoya, Japan), mouse monoclonal anti HLA class I antibody (EMR8-5, Hokudo, Sapporo, Japan), mouse monoclonal anti-MAGE-A antibody (6C1, Thermo Fisher Scientific, Waltham, MA, USA), and mouse monoclonal anti-CCDC62-2 antibody (mAb6212-1) [20,21]. A biotin-labeled secondary antibody was used for detecting the primary antibody, followed by an avidin-biotin complex system (ABC Elite, Vector Laboratories, Burlingame, CA). Immunohistochemical reactions were visualized with 3,3-diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Scoring of immune-related cell infiltration. The degree of immune cell infiltration was analyzed in more

than 10 independent high-power (×200) microscopic fields for each tissue sample. The 5 areas of most abundant distribution were selected. The number of CD4 T cells, CD8 T cells, CD56 NK cells, Treg cells, and PD-1-positive cells were counted both in the tumor stoma and within the cancer nests. Two investigators blinded to the patients' clinical information evaluated all specimens.

Scoring of HLA class I and PD-L1 expression status. The extent of immunohistochemical reactivity for HLA class I and PD-L1 was graded as follows: weak, <25% cells stained; intermediate, 25-75% cells stained; and strong, >75% cells stained.

Statistical analysis. We used the χ^2 test to assess the significance of the associations between neoadjuvant chemotherapy and clinicopathological parameters. The HLA class I and PD-L1 expression patterns between the NAC and nNAC groups were analyzed using the Mann-Whitney test. We calculated disease-free survival (DFS) from the date of the operation to the date of the last follow-up or the date of the patient's event. Differences in the DFS times between patient subgroups were analyzed using the log-rank statistic test. We calculated survival probabilities using the Kaplan-Meier method. In all tests, statistical significance was set at $p < 0.05$.

Results

Clinicopathological characteristics of the OSCC patients. The characteristics of the 18 patients evaluated in this study are summarized in Table 1. We identified no significant differences in terms of preoperative WBC count or lymphocyte count in peripheral blood between the 2 groups. The Kaplan-Meier analysis found no association between the NAC and nNAC groups and the DFS (Fig. 1).

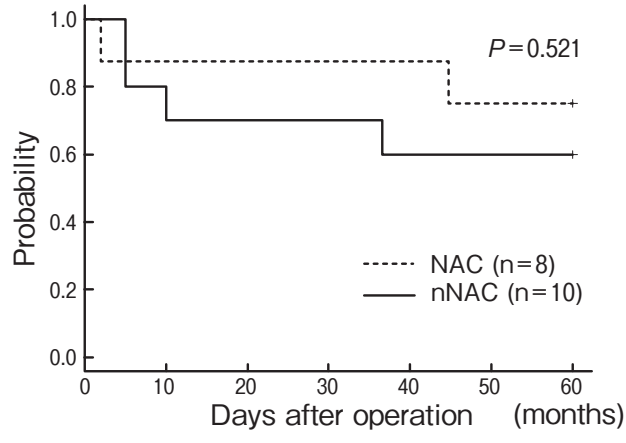


Fig. 1 Disease-free survival (DFS) curves in the NAC (n=8) and nNAC (n=10) groups.

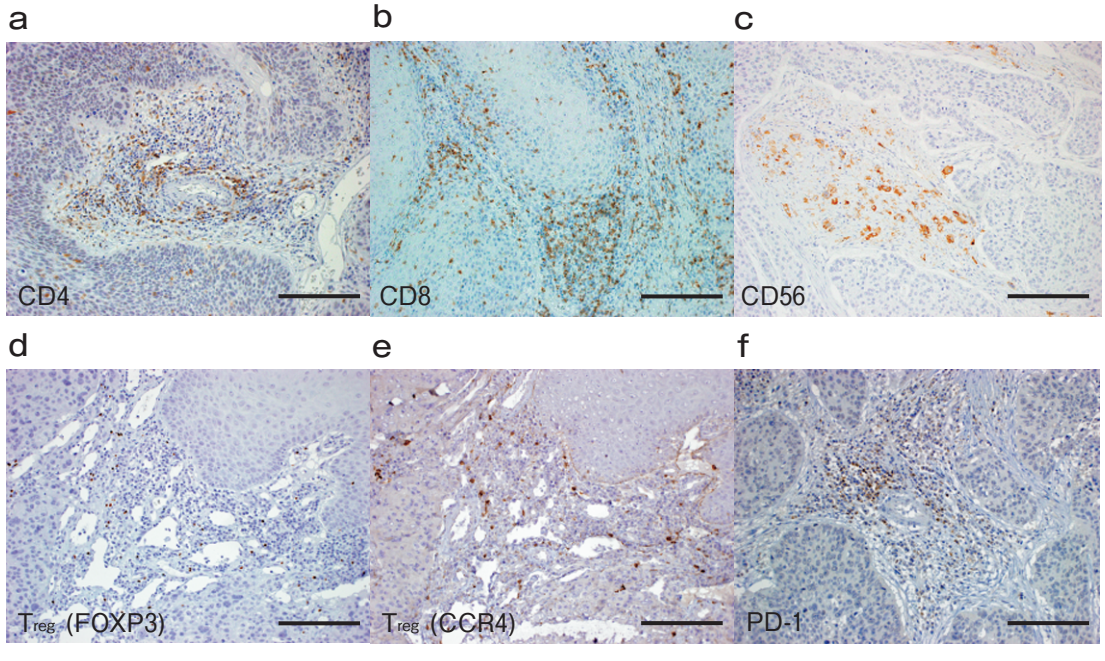


Fig. 2 Representative immunohistochemical staining patterns of formalin-fixed paraffin-embedded primary OSCC sections: (**a**) CD4, (**b**) CD8, (**c**) CD56, (**d**) T_{reg} (Foxp3), (**e**) T_{reg} (CCR4), and (**f**) PD-1. Scale bar, 100 μm.

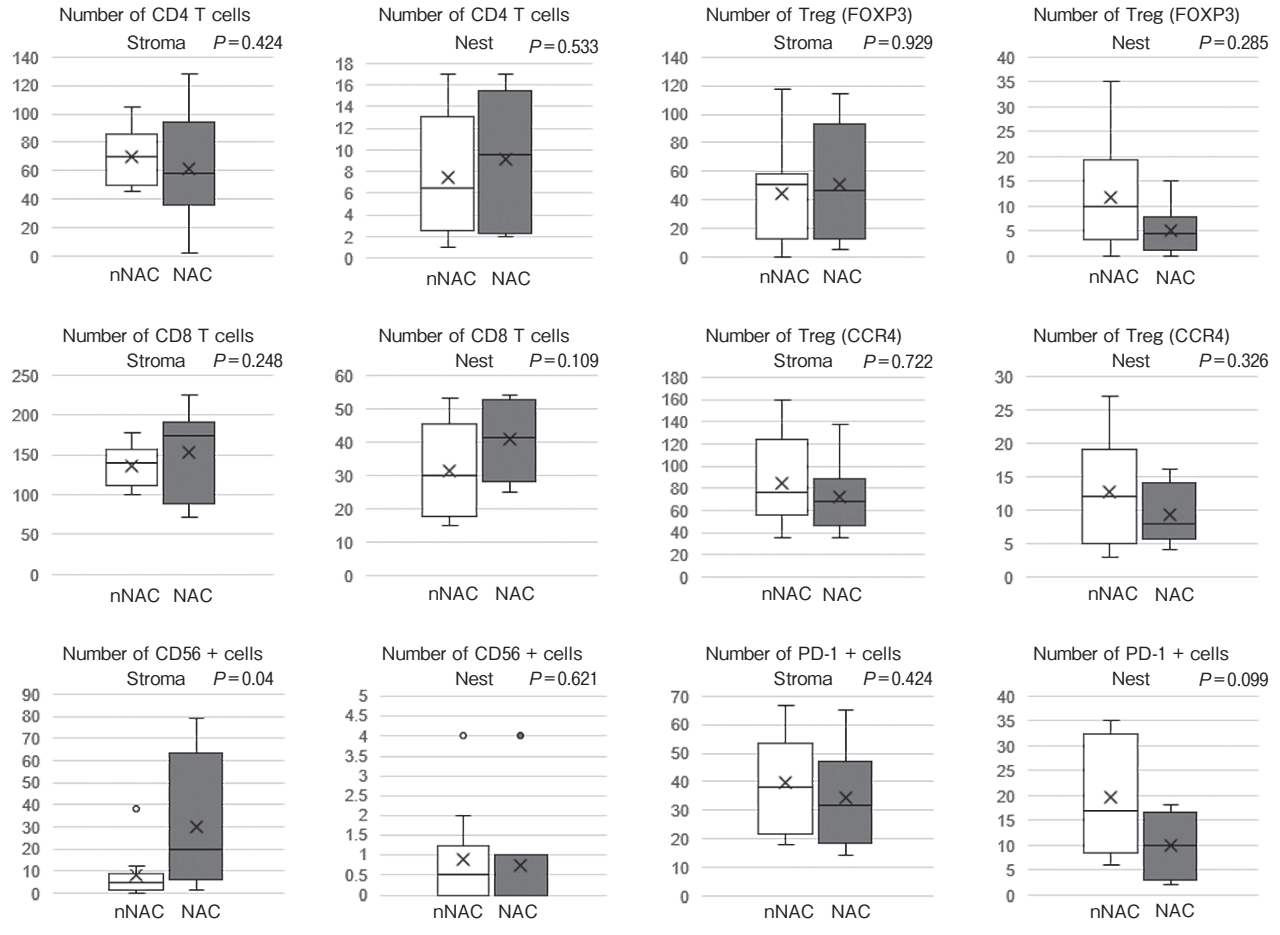


Fig. 3 The degree of immune-related cell infiltration in the tumor microenvironment of the NAC and nNAC groups.

Immunohistochemistry for immune-related cells. We conducted an immunohistochemical analysis to determine the infiltration of immune-related cells such as CD4 T cells, CD8 T cells, CD56 NK cells, Treg cells, and PD-1 positive cells in the cancer specimens. A representative immunohistochemical staining is shown in Fig. 2. Fig. 3 shows the numbers of cells that infiltrated in the cancer nests and mesenchymal stroma. A higher level of CD56 NK cells was found in the stroma than in the cancer nests. Additionally, in the stroma specimens, a significantly higher level of CD56-positive NK cells was found in the NAC group compared to the nNAC group. No significant difference was observed in the other immune-related cells between the 2 groups. Although it was not significant, a decreased number of PD-1-positive cells were found in the NAC group's cancer nest specimens. Thus, we next investigated the expression patterns of PD-L1, which is the major ligand of PD-1, on the cancer cells in the specimens of the cancer nests.

PD-L1 expression. The examination of the PD-L1 protein in the cancer nest specimens revealed that in the nNAC group, 80% (8/10) of the specimens showed intermediate-to-strong expression of PD-L1 protein whereas most of the NAC-group specimens (75%, 6/8) showed weak expression (Fig. 4).

HLA class I expression. Different expression patterns of the HLA class I protein was observed between the 2 groups. As shown in Fig. 5, all 8 of the NAC specimens showed intermediate-to-strong expression. However, 10% (2/10) of the nNAC specimens showed weak expression, suggesting a down-regulation of the HLA class I protein.

CT antigens' expression. The expressions of CT antigens, MAGE-A and CCDC62-2, were evaluated. Staining was heterogeneous in the cancer nest and

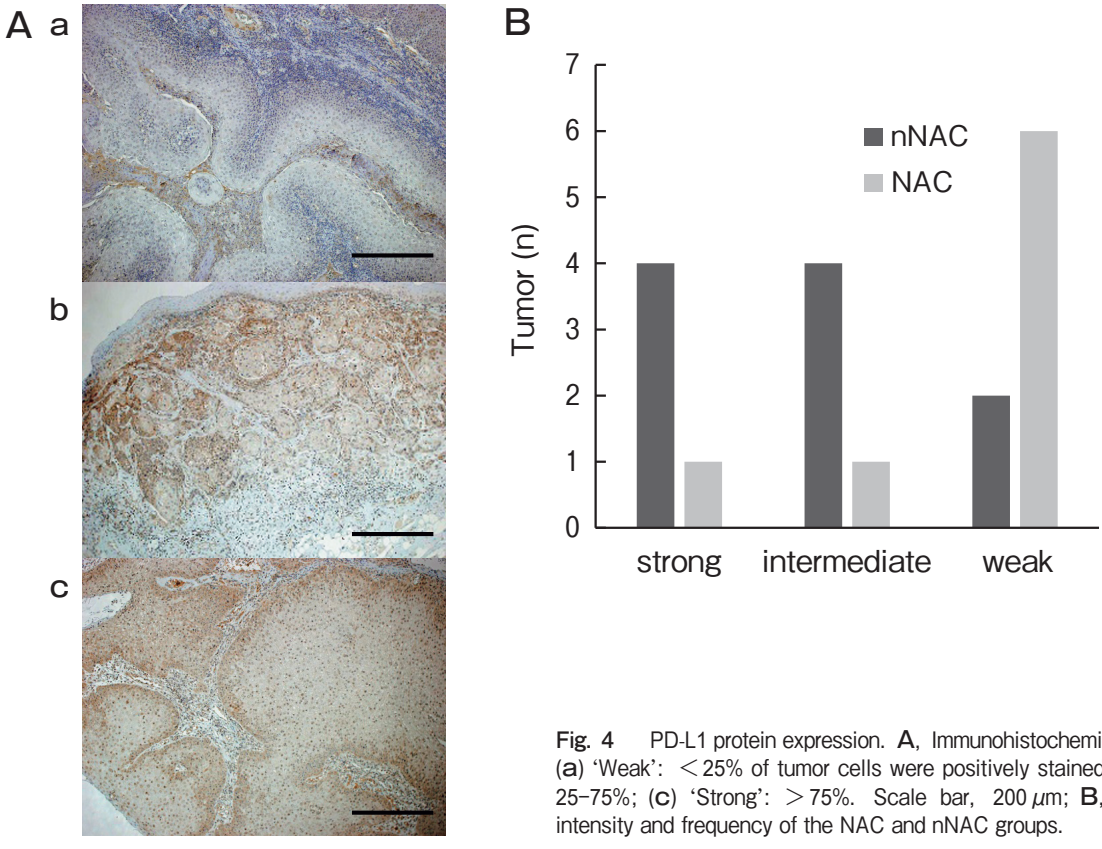


Fig. 4 PD-L1 protein expression. **A**, Immunohistochemical staining patterns. (**a**) 'Weak': ＜25% of tumor cells were positively stained; (**b**) 'Intermediate': 25–75%; (**c**) 'Strong': ＞75%. Scale bar, 200 μm; **B**, PD-L1 expression intensity and frequency of the NAC and nNAC groups.

Table 2 Expression of cancer-testis antigens

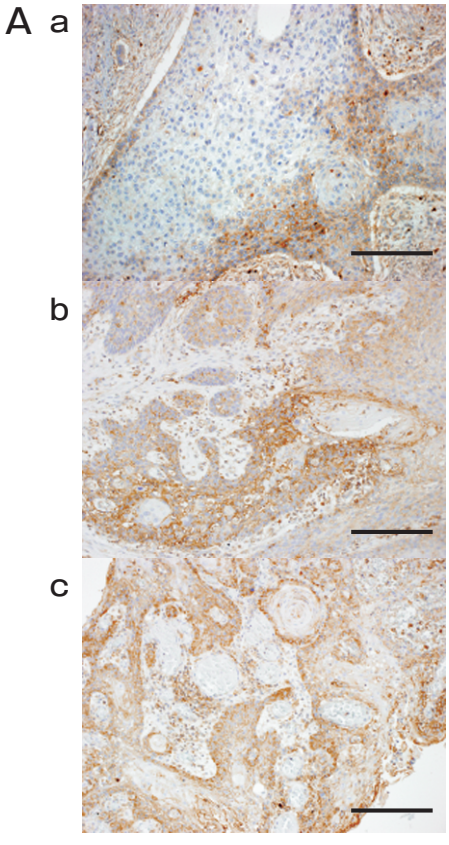
	Antigen	Positive/Total
nNAC (n＝10)	MAGE-A	3/10 (30%)
	CCDC62-2	7/10 (70%)
NAC (n＝8)	MAGE-A	6/8 (75%)
	CCDC62-2	5/8 (62.5%)

localized in the cytoplasm (Fig. 6). In the NAC group, positive staining was observed in 75% (6/8) of the specimens for MAGE-A and 62.5% (5/8) of the specimens for CCDC62-2. In the nNAC group, positive staining was observed in 70% (7/10) of the specimens for CCDC62-2, but only 30% (3/10) of the specimens for MAGE-A (Table 2). Four specimens in the NAC group expressed both antigens (data not shown).

Discussion

Conventional chemotherapeutics are generally thought to kill cancer cells selectively. Accumulating evidence indicates that several chemotherapeutic agents stimulate both the innate and adaptive immune systems [22]. Tsuchikawa *et al.* showed that neoadjuvant chemotherapy with 5-FU and cisplatin increased the intratumoral infiltration of CD4 and CD8 T cells in patients with esophageal squamous cell carcinoma [23]. Nguyen *et al.* demonstrated that multiple subsets of tumor-infiltrating lymphocytes were strong prognostic factors for previously untreated OSCC patients [24]. In the present study, we showed that NAC induced the infiltration of CD4, CD8 T cells and CD56 NK cells into the tumor microenvironment. Conversely, decreased numbers of Tregs and PD-1-positive cells were observed in the NAC group. These findings indicate that conventional chemotherapies may be beneficial in the development of immunotherapeutic protocols for OSCC patients.

Cancer-testis (CT) antigens elicit an immune response in cancer patients and are therefore targets of immunotherapy [20]. In the present study, MAGE-A protein, a prototype CT antigen, was expressed in 3/10 (30%) of the nNAC patients. A high frequency of antigen expression was observed in the NAC patients (6/8,



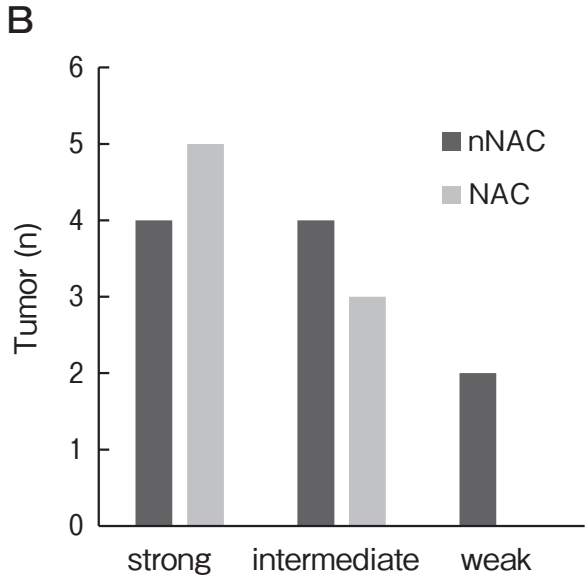


Fig. 5 HLA class I protein expression. **A**, Immunohistochemical staining patterns. (**a**) 'Weak': < 25% of tumor cells were positively stained; (**b**) 'Intermediate': 25–75%; (**c**) 'Strong': > 75%. Scale bar, 200 μm; **B**, HLA protein staining intensity and frequency of the NAC and nNAC groups.

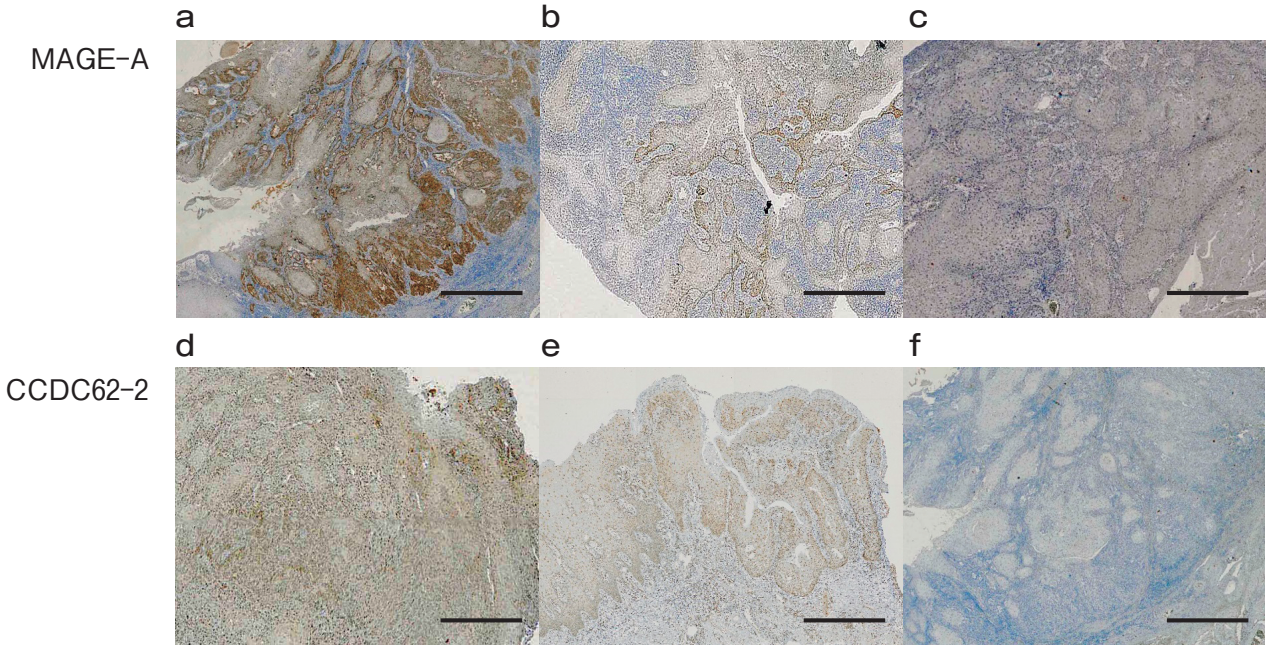


Fig. 6 Immunohistochemical staining of MAGE-A and CCDC62-2 antigens. **a, d**: Strong staining. **b, e**: Weak staining. **c, f**: Negative. Scale bar, 200 μm.

75%), indicating that NAC patients would be eligible for immunotherapeutic treatment.

Immune checkpoints that maintain physiologic self-tolerance were implicated in the down-regulation of anti-tumor immunity [10]. PD-1, a cell surface receptor that is a member of the CD28 family of T-cell regulators, is found on activated T and B lymphocytes as well as NK cells in the microenvironment. Its binding to the ligand PD-L1 on tumor cells causes a down-regulation of T-cell activation, leading to reduced proliferation and to the expression of anti-apoptotic molecules and production of cytokines [25]. PD-L1 expression has been identified in tumor cells of different types of cancer, including OSCC, and is correlated with the tumor grade or prognosis in several types of carcinomas [26]. Blocking the interaction between PD-1 and PD-L1 using monoclonal antibodies has been reported to be a successful treatment for patients with several types of cancer, including melanoma, lung cancer, and kidney cancer [7]. A frequent expression of PD-L1 was observed in OSCCs compared with other cancers [27].

Our immunohistochemical analysis of the infiltration of immune-related cells and PD-1 and PD-L1 protein expression in the surgically resected specimens from OSCC patients with NAC and without presurgical treatment revealed no distinct between-group difference in the degree of immune-cell infiltration, but a decreased number of PD-1-positive cells were observed in the NAC cancer nest specimens. In fact, 75% of the NAC group showed weak PD-L1 protein expression, unlike the nNAC group's intermediate-to-strong PD-L1 protein expression in 80% of the specimens.

The expression of PD-L1 is significantly associated with the histopathological grade in some tumors, including infiltrating ductal carcinoma [28] and urothelial carcinoma [29]. However, the overall prognosis value of the PD-L1 status is controversial. PD-L1 expression is considered a poor-prognosis factor in cancers such as non-small cell lung cancer, renal cell carcinoma, and melanoma [30-32]. In contrast, PD-L1 expression is a good-prognosis factor in colorectal cancer [33]. A recent study revealed that a subset of OSCC patients who were male or had a smoking habit and exhibited high levels of PD-L1 expression has poor clinical outcomes [34]. A smoking history was also marginally associated with PD-L1 expression in non-small cell lung cancer [35]. In our present study, no association was found between the groups of NAC (weak PD-L1 expression) and nNAC (intermediate-to-strong PD-L1 expression) patients and the disease-free survival.

A limitation of our study is the small number of cases. More detailed studies of larger numbers of patients with a variety of clinical-pathological features are needed to address the statistically reliable evidence of immune responses within the tumor microenvironment. In conclusion, our observation revealed that most of the NAC specimens showed down-regulation of the PD-L1 protein, indicating the effectiveness of chemotherapeutic treatment before surgery for OSCC.

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