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Significant association of increased PD-L1 and PD-1 expression with nodal metastasis and a poor prognosis in oral squamous cell carcinoma

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Abstract. Programmed cell death ligand 1 (PD-L1) and its receptor PD-1 are immune checkpoint molecules that attenuate the immune response. Blockade of PD-L1 enhances the immune response in a variety of tumours and thus serves as an effective anti-cancer treatment. However, the biological and prognostic roles of PD-L1/PD-1 signalling in oral squamous cell carcinoma (OSCC) remain to be elucidated. The purpose of this study was to examine the correlation of PD-L1/PD-1 signalling with the prognosis of OSCC patients to assess its potential therapeutic relevance. The expression of PD-L1 and of PD-1 was determined immunohistochemically in 97 patients with OSCC and the association of this expression with clinicopathological characteristics was examined. Increased expression of PD-L1 was found in 64.9% of OSCC cases and increased expression of PD-1 was found in 61.9%. Univariate and multivariate analysis revealed that increased expression of PD-L1 and PD-1 positively correlated with cervical lymph node metastasis. The expression of CD25, an activated T-cell marker, was negatively correlated with the labelling index of PD-L1 and PD-1. Moreover, the patient group with PD-L1-positive and PD-1-positive expression showed a more unfavourable prognosis than the group with PD-L1-negative and PD-1-negative expression. These data suggest that increased PD-L1 and PD-1 expression is predictive of nodal metastasis and a poor prognosis and is possibly involved in cancer progression via attenuating the immune response.

Key words: programmed cell death ligand 1 (PD-L1); programmed cell death 1 (PD-1); oral squamous cell carcinoma; immune checkpoint; metastasis.

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Oral cancer is the eleventh most common cancer worldwide, and the majority is oral squamous cell carcinoma (OSCC)[1,2]. Recent advancements in reconstructive surgery and diagnostic accuracy have improved the survival rate of patients with OSCC[3,4]. However, failure to effectively treat OSCC has fatal consequences because of loco-regional recurrence and distant metastasis. There is growing awareness that therapeutic failure in treating OSCC may be related to malfunction of the immune system.

The successful survival and proliferation of cancer cells involves escape of cancer cells from immune surveillance[5,6]. An immunosuppressive network is established via interactions between cancer cells and immune cells in the microenvironment that leads to cancer cell defence against immune attacks[7]. Previous studies have revealed that immunosuppression by co-stimulatory molecules is involved in the escape from immune checkpoints[8–10]. The most characterized co-stimulatory molecule is cytotoxic T-lymphocyte antigen 4 (CTLA-4) – a member of the immunoglobulin superfamily that plays a key role in the negative regulation of T-cells[11,12]. CTLA-4 also plays a major role in cancer therapy[10,11]. These findings have led to the hypothesis that enhancing the endogenous immune response by CTLA-4 blockade may be an effective anti-cancer treatment. Indeed, the anti-CTLA-4 antibody ipilimumab has been approved as a new treatment for advanced melanoma because of its effects on the overall survival rate[13].

Programmed cell death ligand 1 (PD-L1), also known as B7-H1 or CD274, is another co-stimulatory molecule with important regulatory functions in cell-mediated immune responses[14]. The expression of PD-L1 in tumour cells activates the PD-L1/programmed death 1 (PD-1) pathway by binding to the PD-1 receptor on activated T-lymphocytes and attenuating the immune response[14]. PD-L1 expression has been reported in various types of tumours and is correlated with tumour grade and the prognosis[15–17]. Furthermore, recent studies have demonstrated that blockade of the PD-L1/PD-1 checkpoint is effective in treating melanoma and non-small cell lung cancer[18]. However, few studies have examined the prevalence and prognostic value of PD-L1/PD-1 expression in patients with OSCC[19,20]. This study was performed to examine the association of PD-L1/PD-1 expression with clinicopathological findings and the prognosis in OSCC patients.

Materials and methods

Study design

The study design and methods were approved by the Institutional Review Board of the Centre for Clinical and Translational Research of Kyushu University Hospital. The methods were performed in accordance with the approved guidelines. All patients or their relatives gave written informed consent on admission and prior to their inclusion in the study.

Subjects and samples

Ninety-seven patients with primary OSCC (mean age 64.0 ± 13.6 years; age range 19–88 years), who were diagnosed at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, from January 2006 to December 2010, were enrolled in this study. Following the initial biopsy, specimens were fixed in 4% buffered formalin solution and embedded in paraffin blocks. Specimens were cut into 4-μm-thick sections, stained with haematoxylin and eosin, and examined by experienced oral pathologists to confirm the diagnosis and histological grade. The tumour histological grade was determined according to the World Health Organization classification[21,22]. The extent of the tumour was classified according to the TNM classification, established by the American Joint Committee on Cancer and the International Union Against Cancer (AJCC/UICC)[23]. The mode of tumour invasion was determined according to the Yamamoto–Kohama criteria, as follows: grade 1 = well-defined borderline; grade 2 = cords, less marked borderline; grade 3 = groups of cells, no distinct borderline; and grade 4 = diffuse invasion (4C = cord-like type; 4D = widespread type)[24]. Lymphoplasmacytic infiltration was determined on haematoxylin and eosin-stained specimens according to the Anneroth classification[25]. Patient and tumour characteristics are shown in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm-thick sections prepared as described above. The sections were deparaffinized in xylene and rehydrated in graded ethanol (100%, 95%, 85%, and 75%). For antigen retrieval, the sections were immersed in Dako Target Retrieval Solution (DakoCytomation, Glostrup, Denmark) and autoclaved at 121 °C for 20 min. Endogenous peroxidase activity was then eliminated with 3% hydrogen peroxide for 30 min, and the sections were rinsed twice for 10 min with phosphate-buffered saline (PBS) at pH 7.4. Non-specific protein binding was attenuated by incubation for 30 min with 10% goat serum, and the sections were incubated with each primary antibody overnight at 4 °C. The following antibodies were used: rabbit anti-human PD-L1 monoclonal antibody (clone E1L3N, diluted 1:200; Cell Signaling Technology, Danvers, MA, USA), mouse anti-human PD-1 monoclonal antibody (clone NAT105, diluted 1:100; Abcam, Cambridge, UK), and rabbit anti-human CD25 monoclonal antibody (clone EPR6452, diluted 1:250; Abcam) as a marker of activated T-lymphocytes. The sections were rinsed with PBS twice for 10 min and incubated with secondary antibodies conjugated with a peroxidase-labelled amino acid polymer that reacts with mouse or rabbit IgG, for 1 h at room temperature. After two rinses with PBS for 10 min each, the immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB substrate kit; Nichirei Bioscience, Tokyo, Japan). The sections were counterstained with Mayer's haematoxylin, dehydrated in graded ethanol (75%, 85%, 90%, 95%, and 100%), cleared with xylene, and finally mounted with permanent mounting medium (Malinol mounting medium; Muto Pure Chemicals, Tokyo, Japan). Negative controls included PBS instead of primary antibody.

To evaluate PD-L1 expression in cancer cells at the invasive front, cells expressing PD-L1 were counted in at least three randomly selected areas at 200× magnification. Each area was selected to include at least 100 tumour cells. Two senior pathologists who were not informed of the patients' clinical parameters examined all specimens independently. The percentage of positive cells was calculated as the labelling index, which was obtained by dividing the number of PD-L1 expressing cancer cells by the total number of cancer cells at the invasive front in each area. Results from the two pathologists were averaged and used in the statistical analysis. OSCC patients were then divided into two groups based on a cut-off point (5%) calculated by receiver operating characteristics curve analysis: high PD-L1 expression group and low PD-L1 expression group[26].

Similarly, the patients were also grouped based on the rate of inflammatory cells with PD-1 expression around the cancer nest at the invasive front: those with a rate of ≥30% were assigned to the PD-1-positive group and those with a rate of <30% were assigned to the PD-1-

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