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ANATOMICAL PATHOLOGY

Programmed cell death-ligand 1 expression in oral squamous cell carcinoma is associated with an inflammatory phenotype

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Summary

Phase 2 clinical trials utilising novel anti-PD1/PD-L1 antibodies are being conducted in oral cavity squamous cell carcinoma (OSCC) patients. However, data regarding PD-L1 expression in OSCC is limited.

The aim of this study was to characterise the PD-L1 immunohistochemical expression in OSCC and its association with clinicopathological factors.

Clinicopathological review of 217 patients with OSCC was performed, including quantifying tumour-infiltrating lymphocytes. Immunohistochemistry with PD-L1, CD4 and CD8 was performed.

Forty (18.3%) cases showed PD-L1 expression. Expression was significantly more frequent in females ($p = 0.013$), tongue/buccal mucosal SCCs ($p = 0.05$), and in tumours with a high lymphocytic infiltrate ($p > 0.001$). Intratumoural heterogeneity of PD-L1 expression was observed in 30% of the cases. PD-L1 expression was not significantly associated with disease-free ($p = 0.82$) or overall survival ($p = 0.93$).

PD-L1 expression occurred in a significant minority of OSCC and can be heterogeneous. Frequent PD-L1 expression in OSCCs in females and in tumours with high lymphocytic infiltrate may assist in the selection of patients who may respond to anti-PD1/PD-L1 therapies.

Key words: Immunology; immunotherapy; heterogeneity; OSCC; PD-L1; survival; pathology.

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INTRODUCTION

Surgical resection remains the primary treatment modality for oral cavity squamous cell carcinoma (OSCC) and postoperative adjuvant radiotherapy or chemoradiotherapy are administered when certain adverse histopathological features are present.[1] Approximately 30% of patients with OSCC will die from either locoregional recurrence or distant metastases that cannot be salvaged with standard therapies, as effective second line therapies remain limited.[2] The median survival of patients treated with palliative chemotherapy alone is 8 to 10 months.[3] Despite the strong clinical need, there have been few new effective targeted therapies for patients with advanced OSCC since the introduction of cetuximab, an anti-epidermal growth factor receptor monoclonal antibody, nearly a decade ago.[4]

Several solid tumours show aberrant up-regulation of immunological checkpoints that can dampen T-cell activation and antitumoural immune responses.[5] One such immunological checkpoint is programmed cell death-1 receptor (PD-1), usually expressed by T-lymphocytes, and its ligand-1 (PD-L1), often expressed on macrophages and other immune cells and sometimes on tumour cells.[6] Recently, monoclonal antibodies that can block PD-1 or PD-L1, thus increasing the tumour's susceptibility to antitumoural T cell immune responses, have become available.[7,8] These drugs have shown great promise in pre-treated patients with advanced stage melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma and urothelial carcinoma.[8,9] In most clinical trials, tumoural PD-L1 expression correlated with therapeutic efficacy.[10–12] Clinical trials are currently being conducted to investigate the utility of drugs targeting immune checkpoint molecules in head and neck squamous cell cancers.[7,13,14]

PD-L1 expression has been evaluated in oropharyngeal carcinoma and it has been shown that human papillomavirus (HPV) positive tumours are more likely to show PD-L1 expression as compared to those that are HPV negative.[15–17] However, there are limited data regarding the frequency of PD-L1 expression in OSCC. Similarly, little is known about the association of PD-L1 expression with clinicopathological features of OSCC patients such as age, gender, stage, tumour size, depth of invasion, perineural invasion or lymphovascular invasion. Therefore, we evaluated 217 cases of OSCC using a tissue microarray with immunohistochemistry for PD-L1, CD4, CD8 and *in situ* hybridisation studies for HPV (HPV ISH) to determine the incidence of PD-L1 expression in OSCC, and its association with various clinicopathological factors and survival outcomes.

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MATERIALS AND METHODS

A total of 243 patients with OSCC were identified from the Sydney Head and Neck Cancer Institute database, Sydney, Australia (2001–2012) after approval from the institutional human research ethics committee. The study population consisted of 217 cases after exclusion of patients without archival slides and blocks suitable for detailed histopathological analyses, incomplete clinical follow-up or peri-operative mortality (within 30 days of surgery).

Clinical and follow-up data

Data on patient age, gender, oral subsites, type of surgery, details of post-operative adjuvant therapy, local failure, regional failure or distant metastases and survival were obtained.

Histopathological analysis

The archival slides were reviewed by a pathologist (RG) and scored for histopathological features including tumour size, depth of invasion, pattern of invasion, tumour differentiation, keratin production, lymphovascular and perineural invasion and bone involvement as previously described.[18,19] All tumours were staged utilising the 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (2010) pTNM staging system.[20]

The presence of tumour infiltrating lymphocytes (TILs) was semi-quantified as per the criteria proposed by Busam *et al.* for primary cutaneous melanoma[21] and Klintrup and Huh *et al.* in colorectal carcinoma.[22,23] The TILs were assessed at the infiltrative front of the tumour using a four point scoring system. The TILs were scored 0 if there were no lymphocytes or the lymphocytes did not infiltrate the tumour; patchy infiltration by occasional lymphocytes was scored 1; lymphocytes partially or completely surrounding the tumour islands with focal destruction of tumour islands was scored 2; and diffuse, thick, band-like infiltrate of lymphocytes with destruction of the tumour islands was scored as 3.[21–23] Histopathological evaluation of the factors described above was performed prior to tissue microarray construction and immunohistochemistry.

Tissue microarray (TMA) construction

TMAs were constructed using two cores, each 1 mm in diameter, from the invasive front of the SCC for each case.[24] Highly cellular tumour areas were selected and all attempts were made to avoid areas with haemorrhage, necrosis or excessive keratin deposition.

Immunohistochemistry (IHC)

Whole formalin fixed, paraffin embedded (FFPE) block tissue sections and tissue microarray sections were cut at 3 μm onto Superfrost+ glass slides and stored at 4°C until IHC was performed (<2 weeks).

PD-L1 expression in tumour cells

IHC was performed utilising a Dako autostainer/PT-Link with high pH target retrieval buffer (K8005; Dako, Denmark) as per the manufacturer's instructions. The primary antibody against PD-L1 (E1L3N-XP-Rb mAb; CST#13684) was incubated for 45 min at room temperature at a 1:500 dilution and visualised using the MACH3 Rabbit HRP polymer detection system (M3R531; Biocare, USA) and DAB chromogen kit (BDB2004; Biocare) as per the manufacturer's instructions.

PD-L1 staining of the cell membrane was considered as positive. The staining intensity (0–3) (Fig. 1) and the percentage of positive tumour cells were recorded. An additional 30 whole sections from 16 cases that were negative for PD-L1 immunohistochemistry on TMA sections and 14 cases showing 1+ staining in 5% of the tumour cells on TMA sections were also stained using this protocol to evaluate the heterogeneity in PD-L1 expression in OSCC.

CD4 and CD8 for immune cell infiltrates

Immunohistochemical staining for CD4 and CD8 was performed on 4 μm thick sections from FFPE TMA blocks using Leica Bond III automated staining platform (Leica Biosystems, Australia). Heat-mediated antigen retrieval (100°C) was used at pH 9 for 20 min followed by incubation with mouse monoclonal antibodies to CD4 (clone SP35, 1:50; Cell Marque, USA) and CD8 (clone C8144B, 1:100; Dako) for 15 min. Bond Polymer Refine Detection (DS9800) was used with the standard Leica protocol with an added DAB enhancer step.

The lymphocytic infiltrate was semi-quantified as absent, mild (rare lymphocytes), moderate (focal aggregation of lymphocytes) and severe (diffuse infiltration of lymphocytes) to mirror the histological semi-quantitative assessment of TILs, as described above.[25]

Human papillomavirus (HPV) chromogenic *in situ* hybridisation (CISH)

All cases included in this study were OSCC. However, CISH for HPV DNA was also performed. DNA *in situ* hybridisation on 3 μm TMA sections was performed on fully automated Benchmark Ultra staining platform (Ventana Medical Systems, USA) using Inform ISH iView Blue Plus Detection Kit and Red Counterstain II, (Ventana) according to the manufacturer's instructions. The assay utilised the Ventana HPV III Family 16 ISH Probe cocktail, which hybridised with high risk HPV genotypes including 16, 18, 31, 33, 35, 45, 51, 52, 56, 58 and 66. Cases were classified as either positive or negative. The presence of high risk HPV was scored as positive when either large, homogenous, navy blue precipitate was present (episomal pattern), or discrete, stippled navy blue dots (integrated pattern) were found within the nuclei of malignant cells. Appropriate positive and negative controls were included.

Statistical evaluation

Statistical analysis was performed using Stata version 11.2 (Statacorp, USA). Categorical data were analysed using the chi-square test and normally distributed continuous data using the Independent Samples *t*-test. All p values were two-sided and p values less than 0.05 were considered statistically significant. PD-L1 expression was initially evaluated as a continuous variable representing the percentage of tumour cells demonstrating PD-L1 expression. PD-L1 status was then dichotomised as negative or positive. Disease-free survival was calculated from the date of surgery to date of first disease recurrence, or to the date of death or last follow-up if there was no disease recurrence. For disease-specific survival, patients who died from causes other than OSCC were censored at the time of death. Overall survival was calculated from the date of surgery to date of death or the date of last follow-up. The log-rank test was used to assess differences in survival and survival curves were generated using the Kaplan–Meier method.

RESULTS

The cohort included 130 males and 87 females (M:F = 1.5:1), with a median age of 65 years (range 30–89 years). Post-

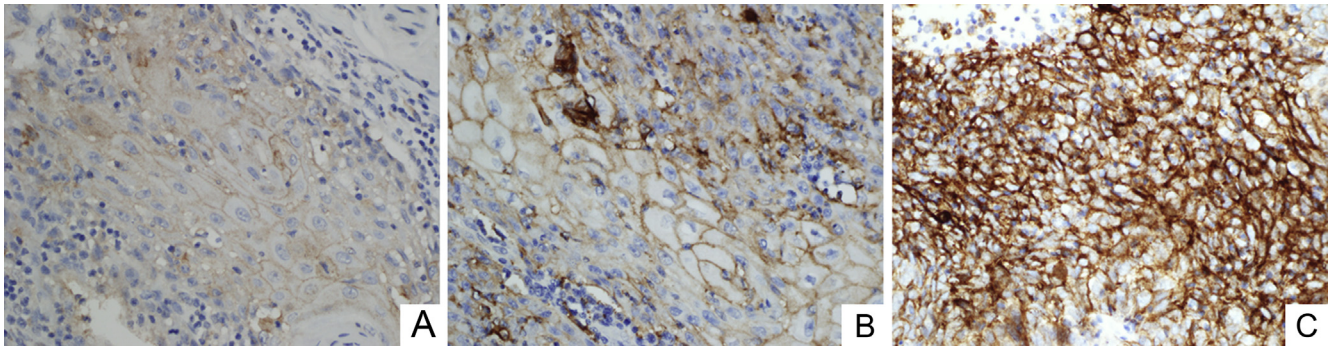

Fig. 1 Immunohistochemistry for PD-L1 by intensity of staining: (A) intensity 1, (B) intensity 2, (C) intensity 3.