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PD-L1 expression in non-dysplastic, dysplastic and oral squamous cell carcinoma samples

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) is an aggressive, highly immunosuppressive cancer with a high mortality rate. The interaction between Programmed-Death 1 (PD-1) (on T cells) and Programmed-Death Ligand 1 (PD-L1) (on tumour cells) within the tumour microenvironment facilitates T-lymphocytic exhaustion and down-regulation. The tumour thus creates an immunosuppressive network that promotes tumour survival and resists innate immunological defence. Elevated PD-L1 expression has been associated with poor prognosis in various other cancers. Blockage of PD-L1 enhances the immune response in a range of tumour-types.

Objectives: To investigate and compare the expression of PD-L1 in non-dysplastic, dysplastic and OSCC samples using immunohistochemistry (IHC).

Methods: IHC was performed on formalin-fixed, paraffin-embedded, archival tissues. The tissues were divided into three groups; non-dysplastic (n = 20), dysplastic (n = 20) and OSCC (n = 20) tissues. Qualitative and quantitative analyses of positively stained cells were undertaken. One-way analysis of variance (ANOVA) was performed to detect statistically significant differences between PD-L1 expression in the dysplastic and OSCC groups when compared against the non-dysplastic group, which served as a control in this experiment.

Results: A higher proportion, darker intensity and a higher immunoreactive score of PD-L1-positive cells was found in the OSCC and dysplastic groups, when compared to the non-dysplastic control group. This difference was statistically significant ($p<0.05$). There was no significant difference between the OSCC and dysplastic tissues.

Conclusion: Greater expression of PD-L1 was correlated to the presence of dysplastic and malignant change. An increase in PD-L1 expression may indicate a disease progression from non-dysplastic tissue to dysplasia and malignancy.

Introduction

Oral cancer is the sixth most common cancer worldwide. Oral squamous cell carcinoma (OSCC) is by far the most common malignant tumour of the oral cavity and it makes up ~2% of all malignancies in New Zealand (Day et al., 2003). Diagnosis and treatment of OSCC have improved in recent years, however, the prognosis of OSCC remains poor (Lin et al., 2015, Maruse et al., 2018). The high mortality rate associated with OSCC could be attributed to late diagnosis and lack of specific biomarkers to complement clinical diagnosis and decision-making (Leemans et al., 2011). Biomarkers such as altered gene sequence and expression levels, or differences in protein structure and function have been associated with various cancers. These markers may be of use in the detection, monitoring and prognostication of cancer. Multiple studies have indicated that molecular markers might serve as potential adjuncts in pathological evaluation of oral tumours to predict risk of relapse following treatment (Shah et al., 2009). However, the relevance of such markers in prognostic determination is not yet clear (Schliephake, 2003).

OSCC is a malignant, highly immunosuppressive tumour of the head and neck region (Hirai et al., 2016). Within the tumour microenvironment, cancer cells interact with host immune cells, creating an immunosuppressive network which promotes tumour growth and resists immunological defences (Maruse et al., 2018). The binding of Programmed-Death Ligand 1 (PD-L1) to the Programmed-Death 1 (PD-1) receptor down-regulates effector cytotoxic T-lymphocytes, which leads to lymphocyte exhaustion and immunological tolerance (Keir et al., 2008). This has been proposed as a mechanism contributing to the immunosuppressive nature of OSCC (Lyford-Pike et al., 2013, Zandberg & Strome, 2014). PD-L1 thus reduces T-cell mediated immune surveillance and therefore facilitates tumour growth and survival (Dong et al., 2002).

Studies have shown that blockade of the PD-1/PD-L1 pathway enhances the immune response in a range of tumours and is thus effective in tumour regression and improving survival (Dong et al., 2002, Maruse et al., 2018, Strome et al., 2003). PD-L1 expression has been detected in several types of cancer including oral cancer, head and neck cancer, glioblastoma, ovarian cancer, renal cell carcinomas, colon cancer, oesophageal cancer, non-small cell lung cancers and melanoma (Chen et al., 2011, Drakes et al., 2018, Konishi et al., 2004, Lin et al., 2015, Müller et al., 2017, Ohigashi, Y., 2005, Shen et al., 2019, Taube et al., 2012, Thomson et al., 2004, Wintterle et al., 2003). Previous research in this field has demonstrated PD-L1's association with poor prognosis (e.g. in lung adenocarcinoma (Yang et al., 2014), renal cell carcinoma (Thompson et al., 2004) and oesophageal cancer (Ohigashi et al., 2005)). Another study showed positive association of epithelial PD-L1 positivity with malignant transformation risk (Yagyuu et al., 2017). Early research has demonstrated the clinical efficacy

of an anti PD-1 monoclonal antibody in treatment of non-small-cell lung cancer (Garon et al., 2015) and melanoma (Hamid et al., 2013, Robert et al., 2015). Current research tends to favour immunotherapeutic approaches to treat the cancerous, malignant state. Comparatively, the immunotherapeutic characteristics and treatment potential of premalignant lesions have been only superficially explored (Young, 2017). A similar trend is evident in OSCC and oral potentially malignant disorders (OPMD)-type lesions, where immunological research into the expression and targeting of molecular biomarkers such as PD-L1 has focussed on OSCC (Wang et al., 2017). Yet, there is only limited information on PD-L1 expression in dysplastic, OPMD lesions (Yagyuu et al., 2017). OSCC-type lesions are often preceded by OPMD lesions, which over a period of time progress to frank malignancy. Therefore, therapeutic strategies that prevent malignant transformation of OPMDs can be expected to reduce OSCC incidence (Wang et al., 2017). Yagyuu et al. (2017) suggest that inhibition of the PD-1/PD-L1 pathway may prevent malignant transformation of OPMD.

Since the PD-1/PD-L1 pathway is an essential mediator of this T-cell exhaustion, it is unsurprising that CD4 and CD8 T cells from patients with *Candida* would exhibit an increase in mean fluorescence intensity for PD-L1, when compared to non-Candidal controls (Jahanshahi & Shirani, 2015). Bakri et al. (2014) did not find any association between the presence of candida and malignant transformation in leukoplakia. However, further research is required to confirm the role of candida infection in the malignant transformation of oral lesions (Shukla et al., 2019).

Since a directly proportional relationship between PD-L1 expression and prognosis is already well-established, this research will instead focus on the correlation between PD-L1 and degree of dysplastic change in oral tissues. The present investigation takes a broader approach, using immunohistochemical techniques to compare PD-L1 expression within and between non-dysplastic, dysplastic and malignant tissue. The relationship of PD-L1 expression and the presence/absence of Candidal infection is another known, but yet comparatively unexplored risk factor for OSCC.

The aim of this study is to investigate the expression of PD-L1 in non-dysplastic oral mucosa tissue, dysplastic oral mucosa and OSCC and the relationship between PD-L1 expression and the presence of Candidal infection within these tissue types.

The tissue samples that we used in this study originate from Sri Lanka, a country where oral cancer makes up 16.5% of all malignancies (Siriwardena et al., 2006), and OSCC incidence is significantly higher when compared to New Zealand. This has allowed us to have a larger size of tissue samples, not commonly available in New Zealand.

Materials and Methods

Sample selection

The University of Otago Human Ethics Committee granted approval for this project on the 17th of May 2019 (reference code H19/061). Samples were selected from 148 Sri Lankan individuals, formalin-fixed, paraffin-embedded (FFPE) biopsy specimens now archived in the Department of Oral Diagnostic and Surgical Sciences at the University of Otago, New Zealand. These biopsy specimens had been collected from patients who presented for treatment of clinically suspicious OPMD/OSCC at the Oral and Maxillofacial Surgery Unit in Teaching Hospital–Karapitiya, a tertiary-level health care institution in Sri Lanka. Prior consent had been obtained for tissue collection and for using them in further research at the University of Otago. After application of the inclusion and exclusion criteria, 60 individual tissue samples were selected. The samples selected for the present study were distinct biological samples of non-dysplastic tissue (n=20), dysplastic tissue (n=20) and OSCC (n=20). Histopathological diagnosis for each of these samples had been confirmed by an oral pathologist.

Sample selection criteria was as follows: Inclusion criteria: 1) Patients who presented for treatment with oral mucosal lesions clinically suspicious of an OPMD/OSCC. 2) Patients who provided informed consent to use their clinical information and remaining tissue samples from initial diagnostic biopsy for further research at the University of Otago. Exclusion criteria: 1) Patients whose clinical records (e.g. DOB, gender) were incomplete; 2) Patients with no suitable tissue remaining (in FFPE blocks) after initial diagnostic histopathology; 3) Patients who had been previously treated for a head and neck malignancy.

Immunohistochemical staining

PD-L1 expression was evaluated using immunohistochemistry (IHC) methods in FFPE archival specimens. The IHC staining for all sections was performed using the BenchMark XT (Ventana® Medical Systems Inc., USA) automated slide stainer. Protocol barcodes were attached to each slide and loaded into the machine. Following automated sequences of deparaffinisation, antigen removal and incubation, pre-diluted 1 µl/ml PD-L1 (1:100 dilution) (i.e. 100µl per slide–1µl of PD-L1 and 99µl of diluent) was manually titrated onto each slide, prior to the incubation and completion of each run. All tissue specimens were then manually counterstained with haematoxylin and cover slipped.

Anti-human PD-L1/mouse monoclonal antibody (Catalogue number: ab210931), manufactured by Abcam (Cambridge, United Kingdom) was used for IHC. The manufacturer's recommended concentration was 5µl/ml. Heat mediated antigen retrieval was performed before commencing the IHC staining protocol. To determine the optimum concentration of PD-L1 to be used in IHC, an optimisation process was carried out using randomly selected SCC samples from within the

study sample as a positive control. The dilution ratio suggested by the manufacturer was used as a reference. This antibody was tested using the Ventana standard protocol (protocol 980). The original PD-L1 antibody was diluted as follows–a 1:50 dilution (i.e. 20µl/ml); a 1:100 dilution (i.e. 10µl/ml) and a 1:200 dilution (i.e. 5µl/ml) were trialled. A 1:100 (10µl/ml) dilution was found to be the optimum dilution for this study, with regard to staining intensity.

Immunohistochemical analysis

Light microscopy (Olympus AX70, Olympus Corporation, Center Valley, PA, USA) was used to evaluate IHC immunostaining both qualitatively and quantitatively, at two magnifications (10x and 20x). General, qualitative assessment of the staining pattern, distribution and intensity was performed by at least two different investigators in the study. Three representative areas of positive-stained cells (hotspots) were first identified under the microscope at 10x magnification and were photographed at 20x magnification (for analysis), using a camera mounted on the microscope (Go-3, QImaging, Survey, BC, Canada).

Positively stained cells were defined as having a brown membrane and intracellular staining. Only cells that were morphologically consistent with an immune cell (e.g. lymphocytes macrophages etc) were counted. In order to exclude false positives, the epithelium or acellular areas were cropped out and excluded from immunohistochemical analysis. Three positive hotspot areas were selected for the purpose of analysis.

Immunohistochemical analysis of PD-L1's immunoreactivity score (IRS) comprised of two parts, namely a) the intensity of staining and b) the proportion score (derived from percentage of positivity). Intensity of staining was measured qualitatively–each photograph was graded thrice by different investigators in the study and an average intensity score calculated. The intensity score of all three hotspots were then averaged to calculate an average intensity score for that particular specimen. Variation in staining intensity observed across the samples were measured qualitatively and assigned values from 0 to 3 (from no staining to high intensity of staining). Proportion score was measured using the software ImageJ (MacOS version 1.51, National Institutes of Health, USA), after excluding areas of epithelium and non-specimen, empty areas.

The final IRS was obtained by multiplying the two values (Proportion Score (PS) and Intensity Score (IS) (Koo et al., 2009 and Lee et al., 2012). For the PS, the number of positive cells in the photomicrograph were counted and the results were expressed as a percentage. A score from 0 to 4 was allocated to each percentage group as follows: 0 = 0%, 1-10% = 1, 11-50% = 2, 51-80% = 3 and 81-100% = 4. The PS was derived from the average value of the three representative photomicrographs taken from each specimen. For the IS, each photomicrograph was categorised using a four-tier scoring system as either negative (0), mild (1), moderate (2) and high (3), as outlined in Table 1. Representative photomicrographs of non-dysplastic (Figure 1B), dysplastic (Figure 1C) and OSCC (Figure 1D) samples have been labelled below to give a representative image of PD-L1 immunostaining for each diagnostic category. Discrepancy of staining and staining morphology between observers were kept below 5% and was resolved at the end of each counting session and referred to the oral pathologist.

To examine the relationship between PD-L1 expression and *Candida*, the final IRS values derived from each photomicrograph were divided into two arbitrary groups. An IRS score of 0 to 3 was considered negative, while a score equal to or greater than four was considered positive (Koo et al., 2009; Lee et al., 2012). All samples were histopathologically examined to detect and record the presence or absence of Candidal hyphae infiltration in the epithelium.

Table 1: Quantitative analysis of IHC and the formulation of the Immunoreactive Score

Percentage of positive cells	Proportion Score (PS)	Intensity Score (IS)	
0%	0	Negative	0
1-10%	1	Mild	1
11-50%	2	Moderate	2
51-80%	3	High	3
81-100%	4		
Immunoreactive score (IRS) = PS x IS			
0-3		Negative	
4-12		Positive	

Statistical analysis of PD-L1 staining

The GraphPad Prism 7 for Mac OS X (GraphPad Software, Inc., USA) software package was used for statistical analysis. One-way analysis of variance (ANOVA) was performed on the IHC data to detect statistically significant differences between PD-L1 expressions for each of the diagnostic groups. Statistical analysis was used to compare expression of PD-L1 in the 3 tissue types. The unpaired student's *t*-test and Mann-Whitney test were used to examine for statistically significant differences in PD-L1 expression between the non-dysplastic samples when compared to the dysplastic and OSCC samples. A p-value of <0.05 indicated data of statistical significance.

Spearman analysis was used to analyse the correlation between PD-L1 expression and the presence of *Candida*, within each diagnostic category (non-dysplastic, dysplastic and OSCC). A p-value of <0.05 indicated data of statistical significance.

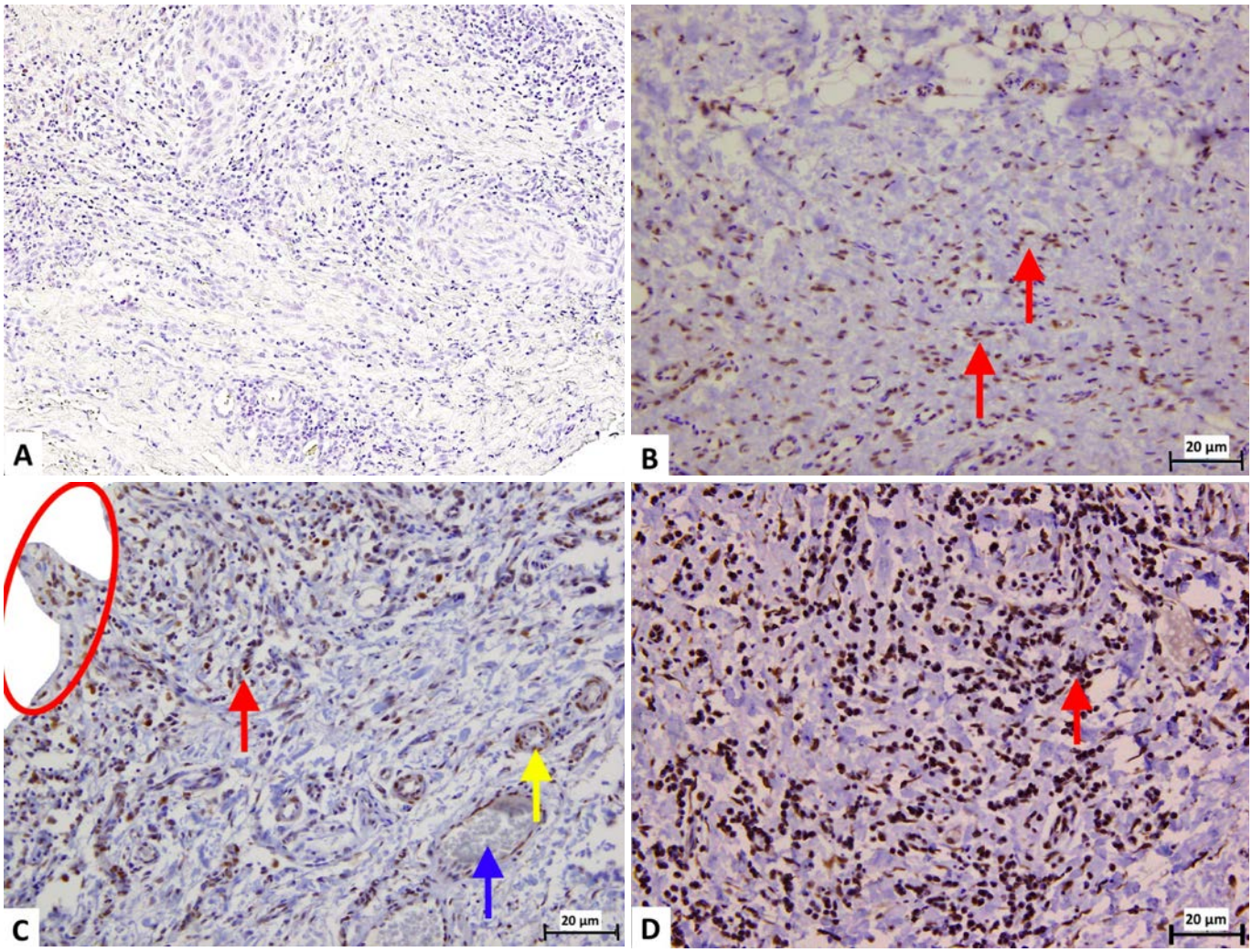

Figure 1 (A, B, C, D): Photomicrograph of IgG control (taken at x10 magnification) and three representative photomicrographs of OSCC, dysplastic and non-dysplastic samples (taken at x20 magnification). **1A:** IgG control at matched concentration to the PD-L1 antibody used in this study. **1B:** A photomicrograph showing low-positive, mild staining intensity of PD-L1 positive lymphocytes (red arrows) sparsely distributed throughout the connective tissue stroma. Lymphocytes were frequently located in the superficial portion of connective tissue. This is representative of photomicrographs taken of non-dysplastic samples in this study. **1C:** A photomicrograph showing low-positive and positive, moderate staining of PD-L1 positive lymphocytes (red arrow) diffusely distributed throughout the connective tissue stroma at a lower concentration than in 1D. Also visible in this photomicrograph are blood vessels (yellow arrow) and glandular tissue (blue arrow). The white area in the top left corner (circled in red) is epithelium that has been removed from proportional analysis. This image is representative of photomicrographs taken of dysplastic samples in this study. **1D:** A photomicrograph showing highly positive, intense staining of PD-L1 positive lymphocytes (red arrow), which are highly concentrated throughout the connective tissue stroma. This is representative of the photomicrographs taken of SCC samples in this study.

Results

Qualitative assessment of IHC on immune cells in the normal mucosa, dysplastic and OSCC samples

A negative isotype IgG control (Figure 1A) was performed to match the concentration of PD-L1 antibody used in this study. Figures 1B, 1C and 1D show three representative photomicrographs of PD-L1 positive immunostaining–one from each of the three diagnostic categories (non-dysplastic, dysplastic and OSCC respectively).

All sample slides were stained successfully except one, dysplastic, sample, which was excluded due to technical error. Results showed an increased number of PD-L1+ inflammatory cells in the stroma of OSCC samples, in comparison to the dysplastic and normal mucosal samples. A majority of the immune cells morphologically consistent with lymphocytes appeared to be randomly located, although some of them were close to the islands and strands of malignant keratinocytes and connective tissue. These cells were seen more frequently in the area close to the epithelium rather than deeper in connective tissue. They were mainly concentrated within the epithelium and around the lumen of blood vessels.

Quantitative analysis of PD-L1 staining

PD-L1 expression in normal oral mucosa, dysplasia and OSCC

1) *Percentage of Positivity of PD-L1 expression*

PD-L1 expression was quantified by the percentage of positively stained cells. In the non-dysplastic group, the mean percentage of positive staining (9.00% [SD = 3.77]) was less compared to the dysplastic group (16.57% [SD = 6.65]). The mean percentage of positive staining in the OSCC group (26.07% [SD = 11.38]) was greater compared to the non-dysplastic group. These differences were statistically significant ($p<0.0001$), as shown in Table 2A and Figure 2A. The differences in percentage of positively stained cells between the dysplastic and OSCC groups were less statistically significant, with a p value of <0.01.

2) *Proportion Score of PD-L1 expression*

Nine out of 20 tissue samples (45%) in the non-dysplastic group had a PS of 2, while 11 of them (55%) had a PS of 1. In the dysplastic group, two of them (10.5%) had a PS of 1, 17 of them (89.5%) had a PS of 2 and one of the samples was excluded as it could not be stained, despite being repeated twice. All values obtained from each group were compared, and details are presented in Table 2B and Figure 2B.

3) *Intensity score (IS) of PD-L1 expression*

In the non-dysplastic group, five out of 20 samples (25%) scored 1 (low positive) and 15 cases (75%) scored 2. In the dysplastic group, excluding the unstained sample, 63% scored 2 (positive), seven samples (37%) scored 3 (high positive). 10 samples

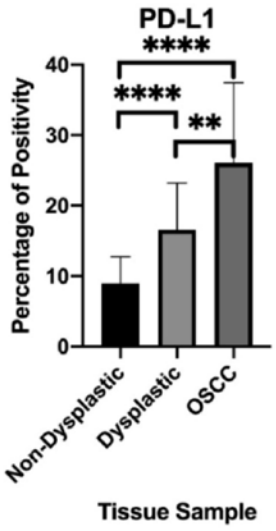

Figure 2A

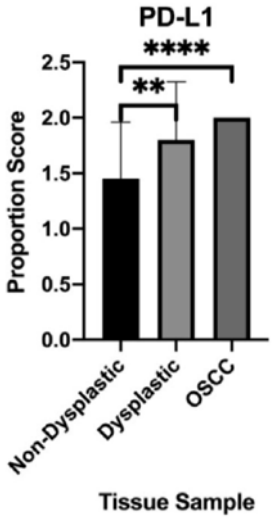

Figure 2B

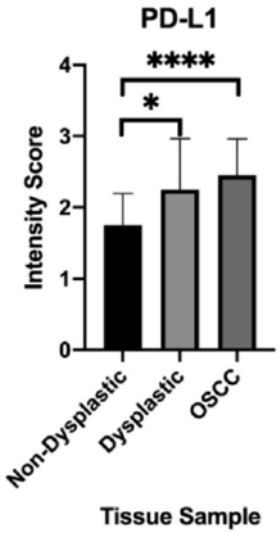

Figure 2C

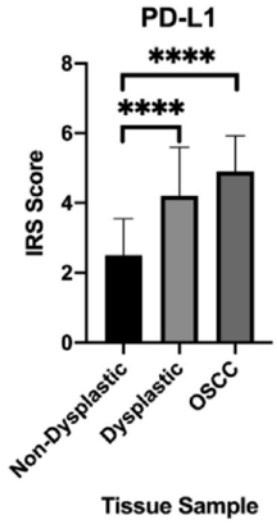

Figure 2D

Figure 2 (A, B, C, D): Bar graphs derived from PD-L1 staining in three diagnostic categories.
2A: Percentage of positivity, (error bars show Standard Deviation) **2B:** Proportion score **2 C:** Intensity score; **2 D:** Immunoreactive score.
Asterisks correspond to p-values (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$).

Table 2(A, B, C, D): Detailed analysis of PD-L1 expression. Tables derived from PD-L1 staining in three diagnostic categories:

2A: Percentage of positive cells

% OF POSITIVITY	Non-Dysplastic (n=20)	Dysplastic (n=19)	Non-Dysplastic (n=20)	OSCC (n=20)	Dysplastic (n=19)	OSCC (n=20)
Mean	9.00	16.57	9.00	26.07	16.57	26.07
Standard Deviation	3.77	6.65	3.77	11.38	6.65	11.38
Standard error of mean	0.84	1.53	0.84	2.54	1.53	2.54
P Value	<0.0001		<0.0001		0.03	
Significant	Yes****		Yes****		Yes*	

Table 2B: Proportion score

PS	Non-Dysplastic (n=20)	Dysplastic (n=19)	Non-Dysplastic (n=20)	OSCC (n=20)	Dysplastic (n=19)	OSCC (n=20)
Mean	1.45	1.90	1.45	2.00	1.90	2.00
Standard Deviation	0.51	0.32	0.51	0.00	0.32	0.00
Standard error of mean	0.11	0.07	0.11	0.00	0.07	0.00
P Value	0.0024		<0.0001		<0.0001	
Significant	Yes**		Yes****		Yes****	

2C: Intensity score

IS	Non-Dysplastic (n=20)	Dysplastic (n=19)	Non-Dysplastic (n=20)	OSCC (n=20)	Dysplastic (n=19)	OSCC (n=20)
Mean	1.75	2.25	1.75	2.45	2.25	2.45
Standard Deviation	0.44	0.72	0.44	0.51	0.72	0.51
Standard error of mean	0.10	0.16	0.10	0.11	0.16	0.11
P Value	0.01		<0.0001		0.90	
Significant	Yes*		Yes****		No	

2D: Immunoreactive score

IRS	Non-Dysplastic (n=20)	Dysplastic (n=19)	Non-Dysplastic (n=20)	OSCC (n=20)	Dysplastic (n=19)	OSCC (n=20)
Mean	2.50	4.42	2.50	4.90	4.42	4.90
Standard Deviation	1.05	1.02	1.05	1.02	1.02	1.02
Standard error of mean	0.24	0.23	0.24	0.23	0.23	0.23
P Value	<0.001		<0.0001		0.99	
Significant	Yes****		Yes****		No	

(50%) in the OSCC group scored 2 and the other 10 samples (50%) scored 3. All values obtained from each group were compared, and details are presented in Table 2C and Figure 2C.

4) *Immunoreactive Score (IRS) of PD-L1 expression*
In the non-dysplastic group, 12 samples (60%) scored 2, six samples (20%) scored 4, and the other two cases (10%) scored 1. In the dysplastic group, there was more variation in IRS values compared to the other two groups. 12 samples (63.2%) scored 4, five samples (26.3%) scored 6, two samples (10.5%) scored 3, and one sample was excluded due to lack of staining. In the OSCC samples, 12 of them (60%) scored 4 and eight of them (40%) scored 6. With these IRS values, all 20 OSCC cases were considered to have positive expression of PD-L1. All values obtained from each group were compared, and details are presented in Table 2D and Figure 2D.

To summarise, there were statistically significant differences in the percentage of positivity, PS, IS and IRS between non-dysplastic and dysplastic groups as well as between non-dysplastic and OSCC samples, as shown in Tables 2A, 2B, 2C and 2D and Figures 2A, 2B, 2C and 2D respectively.

The difference in PD-L1 expression between the dysplastic and OSCC samples varied based on the measure of PD-L1 expression used. The aforementioned tables and figures illustrate statistically significant (albeit weaker) differences between the percentage of positivity and PS of these two disease states. Comparatively, IS and IRS measures did not demonstrate a statistically significant difference between the dysplastic and OSCC groups. As IRS can be considered the summative measure of the other three measures of PD-L1 expression, this study did not find a statistically significant difference in PD-L1 expression between dysplastic and OSCC samples.

Relationship between Candida Positivity and IRS

Spearman analysis was performed to analyse the correlation between positivity for *Candida* and IRS. No statistically significant correlation between *Candida* and PD-L1 expression was observed in any of the three groups. All p-values were higher than 0.05. Therefore, no statistically significant correlation between candidal

Table 3: Spearman analysis comparing positivity of IRS and candida

Diagnostic category	% positive for candida	p-value	Statistically significant?
Non-dysplastic	50%	>0.9999	No
Dysplastic	74%	0.1188	No
OSCC	90%	0.6733	No

and IRS positivity for PD-L1 was detected in the non-dysplastic, dysplastic and OSCC groups. Table 3 shows the percentage of each diagnostic group that was positive for candida, as well as the p-values for all three diagnostic groups.

Discussion

To the authors' knowledge, this is the first study to examine PD-L1 expression in non-dysplastic, dysplastic and OSCC tissues and look at the association of Candida within the groups. Previously published research using IHC for PD-L1 had mainly focussed on the relationships between PD-L1 expression and a) OSCC prognosis and b) OSCC risk factors. To date, there is limited research into PD-L1 expression in premalignant, dysplastic lesions, although targeting PD-L1 in such lesions has been suggested as a preventative treatment modality (Young, 2017).

In the current study, all four measures of PD-L1 expression were elevated in OSCC samples, when compared to non-dysplastic samples and this difference was statistically significant. Elevated PD-L1 levels in OSCC reflect and are comparable to what has been described in the literature regarding PD-L1 expression in other squamous cell carcinomas, specifically head and neck cancer, glioblastoma, ovarian cancer, renal cell carcinomas, colon cancer, oesophageal cancer, non-small cell lung cancers and melanoma (Müller et al., 2017, Wintterle et al., 2003, Drakes et al., 2018, Thomson et al., 2004, Shen et al., 2019, Ohigashi, Y., 2005, Chen et al., 2011, Konishi et al., 2004, Taube et al., 2012). The methodology in many of these studies investigated expression of ligands for the PD-1 receptor (such as B7-H1/PD-L1 and other members of the B7 superfamily) (Zandberg & Strome, 2014, Chen et al., 2011, Dong et al., 2002, Wintterle et al., 2003), which has been shown to down-regulate T-cell activation through PD-1 and thus lead to diminished immune response.

The dysplastic group in this study was also associated with elevated PD-L1 expression, when compared to non-dysplastic samples–and this difference was statistically significant. Since there is comparatively little volume of research into PD-L1 expression in dysplastic, OPMD samples, findings related to this particular diagnostic category appear novel. The clinical relevance of this finding is promising when considered in the context of recent research. Yagyuu et al. (2017) counted subepithelial PD-L1-positive cells in oral precancerous lesions and found that PD-L1 positivity was significantly associated with malignant transformation. This indicates that PD-L1-expressing dysplastic cells in oral precancerous lesions may evade the host immune system. Accordingly, Young (2017) suggests that inhibiting the PD-1/PD-L1 pathway may potentially prevent malignant transformation of OPMD lesions into OSCC.

There were, however, conflicting findings as to whether PD-L1 expression differed between these two disease states (i.e. dysplastic and OSCC), depending upon which measure of PD-L1 expression was used. As such, definitive conclusions regarding differences in PD-L1 expression between these two disease states cannot be drawn from this particular study. Future research could perhaps analyse PD-L1 expression between these two diagnostic categories in greater depth, with a larger sample size.

Unfortunately, there was no clinical follow-up data available regarding the patients in this study. Had this been available, it would have been interesting to assess whether those whose dysplastic lesions exhibited higher PD-L1 expression were more likely to progress to OSCC. This would be an interesting direction for future longitudinal research in the subject of PD-L1 expression.

The findings from this research may have multiple implications in the diagnosis and treatment of OPMD and OSCC. Differential expression of PD-L1 may be used as an adjunctive tool in the differentiation between non-dysplastic, premalignant and malignant lesions. This may assist pathologists and clinicians to better classify and distinguish between OPMD and OSCC lesions and manage these accordingly using PD-L1-targeted therapies in the future. In terms of treatment efficacy, phase 1 clinical trials in a tumour mouse model showed that blocking the PD-1/PD-L1 pathway in head and neck cancer effectively reduces tumour growth and improves survival (Ibrahim et al., 2015). Recent clinical studies in patients with a range of other advanced cancers (non-small cell lung cancer, melanoma, renal-cell cancer, colorectal and prostate cancer) have reliably demonstrated that blocking the PD-1/PD-L1 interaction results in tumour regression (Topalian et al., 2012). This has led to the recent FDA approval of anti-PD-1 antibodies such as Nivolumab and Pembrolizumab for use in second-line treatment of advanced melanoma and non-small cell lung cancer (Topalian et al., 2012, Sui et al., 2018).

The current study detected no correlation between PD-L1 expression and the presence of *Candida*, a suggested etiological risk factor for OSCC (Jahanshahi & Shirani, 2015). The presence of Candidal infection has been associated with T-lymphocytic exhaustion and a suppressive immunophenotype (Spec et al., 2016). To date, research into the immunosuppressive effect of Candidal infections is limited primarily to clinical, systemic data. Spec et al. (2016) found that patients with a Candidal bloodstream infection had an increase in the percentage of cells positive for PD-L1 and PD-1 compared to *Candida*-free controls. De Silva (2018) found that Candidal colonization in oral mucosal lesions had a significant independent association with occurrence of high-risk oral epithelial dysplasia and OSCC. In the

present study, however, PD-L1 expression seems to be independent of the presence/absence of *Candida*.

It should also be noted that there is no established correlation between PD-L1 expression and other, non-Candidal, comparatively better-established, well-known risk factors for OSCC (such as smoking, alcohol consumption and betel quid chewing) (Lin et al., 2015, Müller et al., 2017). Research into PD-L1 expression in HPV, another important factor in the aetiology of oropharyngeal SCC, has produced mixed results, with three independent studies (Lyford-Pike et al., 2013, Badoual et al., 2012, Ukpo et al., 2012) revealing higher expression of PD-L1 in HPV positive compared to negative patients while Müller et al. (2017) showed no correlation.

Existing literature indicates no correlation between the aforementioned oral habits and PD-L1 expression. Both Chen et al. (2018) and Lin et al. (2015) found no statistically significant association between PD-L1 expression and OSCC risk factors such as smoking, betel quid chewing or alcohol consumption. As such, the lack of a correlation between PD-L1 and *Candida* as a risk factor for OSCC reflects current scientific understanding.

PD-L1 expression, as detected through IHC methods, has been demonstrated to be an independent prognostic marker for all major localization of squamous cell carcinoma of the head and neck. Müller et al. (2017) suggest that high PD-L1 expression and activation of the PD-1/PD-L1 pathway in head and neck SCC represents a highly aggressive cancer phenotype and an unfavourable clinical course, independent of tumour origin, stage or grade.

The prognostic value of PD-L1 positivity in other forms of cancer is inconsistent. Most researchers utilized TNM staging to determine prognosis. A strong correlation between poor prognosis and PD-L1 expression in T-cells has been observed in oesophageal cancer (Ohigashi et al., 2005, Chen et al., 2011), renal cell carcinoma (Thomson et al., 2006) and non-small-cell lung cancer (Mu et al., 2011), whereas PD-L1 expression has both negative and positive prediction values in colorectal cancer (Droeser et al., 2013, Liang et al., 2014) and melanoma (Hino et al., 2010, Taube et al., 2012). While the exact prognostic/TNM staging data was not available for the OSCC samples included in this study, a similarly positive correlation was observed between intensity of PD-L1 expression and severity of dysplasia from non-dysplastic to dysplastic to OSCC.

The main strengths of this study included the diagnostic diversity of samples in which PD-L1 expression could be examined. Additionally, the IHC profiler methodology provided us with a quantitative method to measure the proportion of staining in the tissues. The main limitations of the study were the small sample size and the potential for operator error in the various laboratory and analytical stages, particularly pertaining to dilution and loading of PD-L1 onto slides and the qualitative measurements for intensity score. Having the same investigators perform specific tasks (i.e. dilution, loading, cropping out epithelial areas and deriving proportion score) minimised the chance of such systemic, quantitative error. In an effort to minimise estimator bias in the qualitative, intensity score component of this investigation, all investigators underwent calibration prior to evaluating intensity score. The intensity score of each photomicrograph was assessed thrice by three, independent investigators before being averaged to derive an intensity score for that particular hotspot/image. The intensity score of all three hotspots were then averaged to calculate an average intensity score for that particular specimen. The main strengths of the study pertain to the quantitative measurements of PD-L1 expression in non-dysplastic, dysplastic and OSCC samples from a standardised cohort.

It would be valuable to investigate further within the dysplastic and OSCC categories, and determine whether within each of these categories, a similarly positive relationship exists between PD-L1 expression and degree of dysplasia, severity/TNM staging/prognosis of OSCC. Further research should investigate whether cytokines blocking the PD-1/PD-L1 pathway differ in efficacy when treating premalignant and OSCC lesions. It would also be beneficial to repeat this experiment with similar samples from other countries to increase the global relevance of these findings. Furthermore, as the prognostic value of PD-L1 expression in melanoma is still controversial, performing a similar experiment with melanoma would be of particular value to New Zealand, when considering the high national incidence of melanoma.

Conclusions

Cells histomorphologically consistent with T-lymphocytes were the predominant PD-L1-positive cell type detected in these oral mucosal samples. PD-L1 expression was significantly higher in dysplastic and OSCC samples when compared to samples of non-dysplastic oral mucosa, and this difference was statistically significant. Therefore, the hypothesis was partially upheld. These results suggest that PD-L1 expression can be used as an adjunctive tool in the differentiation between non-dysplastic, premalignant and malignant lesions, and that the PD-1/PD-L1 pathway has potential for use as a therapeutic target.

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