DIFFERENTIAL EXPRESSION OF PROGRAM DEATH LIGAND 1 IN PROLIFERATIVE
VERRUCOUS LEUKOPLAKIA

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A thesis submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Adams School of Dentistry (Oral and Maxillofacial Pathology).

Chapel Hill
2019

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ABSTRACT

Si On Lim: Differential Expression of Program Death Ligand 1 in Proliferative Verrucous
Leukoplakia
(Under the direction of Ricardo Padilla)

The objective of the study was to evaluate the expression of program death ligand 1 (PD-L1) in lesions of proliferative verrucous leukoplakia (PVL). Eight PVL patients with both low- and high-risk lesions were selected. Their archived biopsy specimens were retrieved from the UNC School of Dentistry Oral Pathology Laboratory. Amalgam tattoo biopsy specimens were selected as control. Immunohistochemistry for PD-L1 was performed on tissue sections. The proportion of epithelial cells expressing PD-L1 was scored.

The interobserver agreement for PD-L1 scoring was very good (ICC=0.94). All sixteen controls showed no PD-L1 expression. One of 12 low-risk lesions and 15 of 18 high-risk lesions showed ≥1% PD-L1 expression. There was an association between the risk groups and PD-L1 expression (p=0.004), and the odds ratio of high-risk lesions having ≥1% PD-L1 expression was 54. Our results suggest that anti-PD-1/PD-L1 therapy may be beneficial for patients with PVL who develop high-risk lesions.

To my husband, you are teaching me that happiness is what you make, not what you find. I could not have done this without you.

ACKNOWLEDGEMENTS

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CIS	Carcinoma-in-situ
CMH	Cochran-Mantel-Haenszel
EBV	Epstein-Barr virus
FFPE	Formalin-fixed and paraffin embedded
H&E	Hematoxylin and eosin
HG	High-grade
HPV	Human papillomavirus
ICC	Intraclass correlation coefficient
IHC	Immunohistochemistry
ISH	In-situ-hybridization
LG	Low-grade
MTR	Malignant transformation rate
OE	Oral erythroplakia
OED	Oral epithelial dysplasia
OL	Oral leukoplakia
OPMD	Oral potentially malignant disorder
OPSCC	Oropharyngeal squamous cell carcinoma
OR	Odds ratio
OSCC	Oral squamous cell carcinoma
PD-1	Program death 1
PD-L1	Program death ligand 1
PVL	Proliferative verrucous leukoplakia

SCC   Squamous cell carcinoma

SOD   School of Dentistry

VC   Verrucous carcinoma

WHO   World Health Organization

CHAPTER 1: ORAL POTENTIALLY MALIGNANT DISORDERS

Introduction

Oral epithelial dysplasia (OED) is a histologic diagnosis demonstrating irregular maturation pattern of the epithelium. On biopsy, OED may be found in several conditions of the oral cavity that have the potential to progress to malignancy. The World Health Organization (WHO) designates these conditions as "oral potentially malignant disorders" (OPMDs) and defines them as "clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal mucosa."[1] Correctly recognizing and managing these entities based on their clinical and histological presentation is essential in preventing the malignant transformation. Here, we first describe the features of OED and its subset, HPV-associated OED. We then describe the following OPMDs: oral leukoplakia (OL), oral erythroplakia (OE), and proliferative verrucous leukoplakia (PVL).

OED is graded into three categories: mild, moderate, or severe dysplasia.[1] The grading correlates with the risk of malignant transformation.[1,2] A subset of OED have been found to have presence of human papillomavirus (HPV) in the epithelium, therefore, is called "HPV-associated OED".[3,4] As mentioned above, OED can be detected histologically in many clinical entities that are characterized as OPMDs. OL is an OPMD that occurs as a white plaque that cannot be explained by a specific condition and requires a biopsy for a definitive diagnosis.[1] OE appears as a red patch or plaque that is frequently associated with a higher degree of epithelial dysplasia; therefore, is associated with a higher rate of malignant transformation compared to that of OL.[1,5] Lastly, PVL is a rare pre-malignant condition with a predilection for older

females.[6] It is characterized by persistent, spreading and multifocal leukoplakic lesions with a high rate of malignant transformation.[6] The following sections describe these entities in more detail.

Oral Epithelial Dysplasia

OED is a histopathological diagnosis characterized by epithelium that demonstrates the cytological and architectural changes presented in Table 1.1.[1,7] OED may be seen in several OPMDs such as OL, OE, and PVL on biopsy.[1] Most commonly, the degree of dysplasia is graded as mild, moderate, and severe, as recommended by the WHO.[1] Mild dysplasia demonstrates minimal abnormal architectural and cytological changes in the lower third of the epithelium.[2,7,8] Moderate dysplasia shows architectural and cytological abnormalities extending into the middle third of the epithelium.[2,7,8] Finally, severe dysplasia displays abnormal architectural and cytological alterations extending beyond two-thirds of the epithelium, and the terminology is interchangeable with "carcinoma-in-situ (CIS)".[1,2,7,8] These grades may be upgraded based on the degree of architectural and cytologic atypia as well as the architecture of the connective tissue interface.[1] It is generally postulated that OED progresses from mild to moderate to severe dysplasia before developing squamous cell carcinoma (SCC), as seen in cervical dysplasia and carcinomas.[9] However, this idea has not been confirmed and has been challenged in the oral cavity since there is evidence of mild dysplasia and even nondysplastic lesions developing into SCC.[9,10]

Although the three-tier grading system has been established for some time, there is a significant variability in grading of OED between and within pathologists due to subjectivity.[7] Reviewing several studies that have investigated inter- and intraobserver agreement for the three-

tier grading system, the interobserver agreement ranges from poor to moderate while the intraobserver agreement ranges from slight to moderate.[11–14] To decrease the variability of the three-tier grading system of dysplasia, Kujan et al.[15] proposed a binary grading system and compared the interobserver agreement to that of the three-grade system. The authors graded dysplasia as "high-risk" if at least four architectural changes and five cytological changes were observed and as "low-risk" if less than four architectural and less than five cytological changes were observed. Their study, along with several subsequent studies, demonstrated slight improvement in interobserver agreement when the binary grading system was utilized.[14–16]

Despite the issues with reproducibility using the three-tier grading system of OED, it is still the best predictive factor for malignant transformation.[2] In a meta-analysis, Mehanna et al.[17] analyzed fourteen studies and reported the pooled mean malignant transformation rate (MTR) of 2.1% in OED. They found an association between the degree of dysplasia and the MTR where the MTR was 10.3% for mild/moderate dysplasia and 24.1% for severe dysplasia.[17] A study by Sperandio et al.[18] supported this finding and reported MTR of 6% for mild dysplasia, 18% for moderate dysplasia, and 39% for severe dysplasia. Similarly, Kujan et al.[15] demonstrated that the binary system can also predict malignant transformation of dysplasia with an accuracy rate of 82%. However, the binary system requires further studies to validate its use according to the WHO.[1]

There is lack of consensus on the most effective management of OED at this time.[19,20] Various treatment methods have been employed that are both surgical and nonsurgical. Surgical methods include local excision, CO_2 laser ablation, and electrodessication.[19] Due to lack of randomized controlled trials data and various contradicting outcomes, no one method has been found to be superior to the others.[19,20] Despite these facts, Mehanna et al.[17] found in their meta-

analysis that OED that was treated with surgical excision had significantly lower MTR (5%) than OED that was not surgically removed (15%). This finding is supported by a recent study which also demonstrated that wide excision and/or ablation is more effective than observation in preventing malignant transformation and recurrence of oral cavity, oropharyngeal, and laryngeal dysplasia.[21] Several topical and systemic agents have been investigated for nonsurgical treatment of OED. The World Workshop on Oral Medicine reviewed studies that utilized topical bleomycin, systemic retinoic acid, and systemic lycopene for OED management.[19] The review concluded that there is lack of evidence that these agents prevent malignant transformation.[19]

Based on algorithms proposed by various authors, Awadallah et al.[22] proposed the following treatment and follow-up algorithm for OED.[22–24] For mild dysplasia, excision and/or laser ablation is recommended for high suspicion lesions, while a conservative management is recommended for low suspicion lesions.[22–24] For moderate and severe dysplasia, excision with clear margins ($\leq$ 2mm for mild and 5 mm for severe) and laser ablation is recommended.[22–24] Even after treatment, mild and moderate dysplasia should be followed every 6 months initially, then extending to yearly.[22–24] Severe dysplasia should be followed every 3 months initially, then extending to every 6 months.[22–24] Regardless of the grade of dysplasia, all patients with OED should be followed over their lifetime.

HPV-Associated Oral Epithelial Dysplasia

A subset of OED was found to be associated with human papillomavirus (HPV).[1] HPV has become a well-established risk factor for a subset of head and neck SCC, especially for the oropharyngeal anatomic site.[25] The incidence of HPV-associated head and neck cancer has increased significantly in the past several decades.[25,26] HPV-positive oropharyngeal SCC

(OPSCC) is associated with a better prognosis compared to HPV-negative OPSCC.[25] Several meta-analysis and systematic review studies have reported association between HPV and a subset of OSCC.[27–30] However, prognosis of HPV-positive OSCC is still unclear at this time. Kansy et al.[31] reviewed several studies regarding HPV and OSCC and found both favorable and unfavorable impacts of HPV on prognosis. In both OPSCC and OSCC, high-risk HPV-16 is the most common genotype detected.[25,27–29] Currently, immunohistochemical (IHC) analysis for p16 protein along with HPV-16 in-situ-hybridization (ISH) on formalin-fixed and paraffin-embedded tissue are routinely utilized techniques for detection of HPV in OPSCC and OSCC.[25]

Unlike OPSCC and OSCC, the evidence for the association between HPV and OED is limited but not absent. In a review of the literature, Miller and White found that of nineteen studies that investigated HPV presence in OED, 18.5% of cases detected HPV, and HPV-16 and -18 were the most frequent genotypes.[32] A meta-analysis reported that the pooled probability of detecting HPV in OED was 26.2%.[29] In addition, Jayaprakash et al.[33] reported a 25.3% overall prevalence of HPV-16/18 in OED in a meta-analysis.

Several studies provide clinical features of HPV-associated OED. The lesions appear as leukoplakic, erythroleukoplakic, or erythroplakic plaques or patches with occasional papillary or verrucous surfaces.[34,35 3,36] Tongue and floor of the mouth are the most common sites reported, but buccal mucosa, gingiva, lips, and palate can also be affected. [3,4,32,34–36] Median age is often reported in the sixth decade of life, and males are affected more often than females with one study reporting a 7.8:1 male to female ratio. [3,4,32,34–36]

Only a few studies have looked at the histological features of HPV-associated OED. In addition to conventional dysplastic changes, karyorrhectic and apoptotic cells are frequently observed throughout the epithelium of HPV-associated OED, which is the feature that can help

to distinguish this entity from conventional OED.[3,34,35] Other findings of the epithelium include

parakeratosis and/or hyperkeratosis, acanthosis, mitotic-like structures, multinucleated cells,

koilocytes, and dyskeratotic cells.[3,4,35,36] McCord et al.[4] also reported loss of squamous

differentiation and a basaloid appearance as the distinguishing features of HPV-associated OED.

Currently, there is not enough evidence regarding prognosis or malignant transformation rate of

HPV-associated OED due to insufficient follow-up data.

Oral Leukoplakia

The current WHO definition of OL is "a clinical term used to describe white plaques of

questionable risk, once other specific conditions and other oral potentially malignant disorders

have been ruled out, which normally requires biopsy."[37] As the definition suggests, the

diagnosis is based on exclusion of other white lesions of oral cavity that have known etiology,

such as oral lichen planus, leukoedema, and tobacco pouch keratosis. The terminology should be

used as a clinical description until it is replaced with the histopathological diagnosis after biopsy.

The global prevalence estimate of OL is 1.49% to 2.5%, and it is higher in males than

females by 3.2 to 4.8 fold.[38] The lesion generally occurs in the fifth decade of life or later.[39–41]

Although any oral mucosa can be affected, the most common sites are gingiva and buccal

mucosa.[41–44] The most significant risk factors are attributed to tobacco use and alcohol

consumption.[2,39,41,45,46] Consequently, OL is much more common in smokers than nonsmokers,

with heavier smokers developing larger and greater number of the lesions.[39,47]

An early lesion appears as a thin and slightly elevated white plaque with or without

distinct borders.[40,45] The lesion may progress to a thickened white plaque with fissures,

especially in smokers.[39,40] OL can be divided into two types based on their clinical appearance:

homogenous leukoplakia and nonhomogeneous leukoplakia.[45] The nonhomogeneous

leukoplakia can be further divided into erythroleukoplakia (having mixed red areas), nodular, or

verrucous leukoplakia based on the surface coloration or architecture.[45]

OL is considered potentially malignant due to the increased risk of transformation into

oral squamous cell carcinoma (OSCC). The estimated mean malignant transformation rate is

14.9%.[48] Risk factors associated with transformation are the presence of epithelial dysplasia,

especially higher degrees of dysplasia, nonsmoker status, nonhomogeneous leukoplakia, lesion

greater than 200 mm^2 in size, female gender, persistence, location on the tongue and/or floor of

the mouth, and presence of *C. albicans*.[48,49] Nonhomogeneous lesions and presence of dysplasia

are generally regarded as the greatest risk factors for malignant transformation[40,49]. Malignancy

may develop at the site of existing leukoplakia or at another site in the oral cavity.[40,49] The most

common sites of malignant transformation are the tongue and the combination of tongue and

floor of the mouth.[48]

Most OLs are benign upon histopathological examination, displaying hyperkeratosis with

a thickened layer of ortho- or parakeratotic surface or acanthosis.[39,40] A small number of them

show dysplastic changes in the epithelium, ranging from mild to CIS and rarely SCC.[40,41]

Lesions of the floor of the mouth and the tongue have the highest likelihood of harboring

dysplasia or carcinoma.[41]

The initial management of OL is incisional biopsy of persistent lesions after eliminating

any potential causative sources, such as trauma and tobacco use.[20,22,39,50] If dysplasia is not

present, van der Waal and Axéll[50] recommend a follow-up of every 6 to12 months. However,

Holmstrup et al.[51] argue a follow- up of every 3 to 6 months regardless of the dysplasia status.

The surveillance should be life-long due to the risk of malignant transformation regardless of the

presence of dysplasia.[23,50] Some OLs have been reported to regress spontaneously.[52] If

dysplasia is present, then the management should depend on the grade of dysplasia, which will

be discussed in a later section.

Treatment methods for OL include surgical excision and/or carbon dioxide (CO_2) laser

treatment. In a study by Holmstrup et al,[53] the surgical treatment carried a recurrence rate of 11%

for both homogenous and nonhomogenous OL.[53] For a treatment with carbon dioxide (CO_2)

laser, the recurrence rate ranged from 3.1% to 40.7% in a systematic review.[54] Currently, no

effective non-surgical treatment has been found to prevent the malignant transformation and

recurrence of OL.[55]

Oral Erythroplakia

Like OL, oral erythroplakia (OE) is a clinical term describing a red patch or plaque after

exclusion of other oral conditions that can be classified definitively, such as erythematous

candidiasis or erosive lichen planus.[1] Due to its rarity, not as much data is available.[56] The

prevalence of OE is between 0.02% and 0.83%, and it is, in general, a lesion of middle-aged to

elderly with no gender predilection.[56]

The lesion appears red and flat with a smooth and velvety, but occasionally granular or

nodular, surface that may be depressed.[45,49,56] The borders are usually well-defined but irregular

in shape.[45,56] Any oral mucosal surface can be affected, but the soft palate is consistently

reported to be the most common site.[39,45,56] Although the definitive etiologic factors are

unknown, tobacco and alcohol are considered to be the main risk factors in developing OE.[49,56]

The clinical significance of OE is its association with OED, CIS, or SCC upon

histopathologic examination.[5] Shafer and Waldron reported that of 65 biopsy specimens of OE,

51% were invasive carcinoma, 40% were CIS or severe epithelial dysplasia, and 9% were mild

or moderate epithelial dysplasia.[5] When Yang at al. examined excised OE lesions from 84

patients, 4% of the lesions were SCC, 24% were high grade epithelial dysplasia or CIS, 49%

were low or intermediate grade epithelial dysplasia, and 24% were hyperplasia.[57] Conversely,

the majority of early asymptomatic OSCCs appear erythroplakic or mostly erythroplakic.[58,59]

Because of the high incidence of severe epithelial dysplasia or CIS in OE, the malignant

transformation rate of OE is estimated from these entities, and is reported to be from 3.2% to 50%

in multiple studies from various countries. [2,56] However, these studies also included lesions that

appeared leukoplakic. Therefore, the true malignant transformation rate of OE is difficult to

estimate.

Biopsy is crucial in management of OE due to its frequent association with epithelial

dysplasia, CIS, and SCC, followed by complete excision, if appropriate, based on the

histopathological diagnosis.[22,45,49] There are only few studies that investigated treatment

outcome of OE. Yang et al.[57] observed 17% recurrence rate after excision with carbon dioxide

laser, and a lesion area greater than 80 mm^2 was a predictive factor. Vedtofte et al.[60] found that

40% of the patients with OE experienced recurrence after surgical excision. Currently, there is

lack of investigation into non-surgical management of OE.

Proliferative Verrucous Leukoplakia

In 1984, Silverman et al.[42] reported findings in 257 patients with oral leukoplakia

followed over a mean of 7 years to study the factors associated with malignant transformation.

During the study, a subset of oral leukoplakia was noted to have distinctive characteristics with a

high rate of malignant transformation.[6,42] Hansen et al.[6] followed 30 patients with these

characteristics for an average of 6.1 years, further describing the condition and naming it "proliferative verrucous leukoplakia" (PVL) in 1985. According to the authors, PVL initially presents as a leukoplakic lesion diagnosed as hyperkeratosis upon biopsy. The lesions persist and spread, or become multifocal over time, with some of them developing an exophytic and warty appearance. Eventually, verrucous carcinoma or squamous cell carcinoma arises in the majority of the lesions.

Since the initial description of PVL by Hansen et al.,[6] several studies have validated and further described the entity.[42,61,62] PVL is a premalignant condition that affects an older population, occurring predominantly in the seventh decade of life.[63] A recent systematic review reported a pooled female-to-male ratio of 2.5, which is less striking than the initial ratio of 4 reported by Hansen et al.[6,63] Leukoplakic lesions are the most commonly observed clinical feature, especially in the early stage.[6,61,64,65] Over time, these lesions progress to become widely spread and/or multifocal, with some of them developing exophytic, verrucous architecture.[6,64] The lesions may take on erythematous, speckled leukoplakic, erosive, or fissured appearances as well.[6,61,66,67] Gingiva, buccal mucosa and tongue are the most common sites affected by PVL.[63] One study observed that verrucous and erythematous appearances are most common on gingiva.[66]

Histopathologically, the early lesions appearing as OL are most frequently hyperkeratosis.[64,66] The lesions then then progress toward malignancy, advancing through the spectrum of dysplasia.[42,61,62] Hansen et al.[6] proposed 10 histological stages of PVL. They gave each stage a numerical grade with the following histological designations: grade 0, normal oral mucosa; grade 2, hyperkeratosis with little or no dysplasia; grade 4, verrucous hyperplasia with little or no dysplasia; grade 6, verrucous carcinoma; grade 8, papillary squamous cell carcinoma;

and grade 10, less differentiated squamous cell carcinoma. The intermediate grades, the histopathology that fit between the grades designated with even numbers, were given odd numbers 1 through 9.[6] In 1999, Batsakis et al.[68] proposed to reduce the number of stages to 4: clinical flat leukoplakia without dysplasia, verrucous hyperplasia, verrucous carcinoma, and conventional SCC. These histopathological staging systems either understate or omit dysplastic changes. However, subsequent studies have frequently described dysplasia in leukoplakic lesions of PVL.[64,66,69–72] The prevalence of dysplasia in PVL has been found to be 47.7%.[63] Based on these findings, the histopathology of PVL includes hyperkeratosis, verrucous hyperplasia, dysplasia, verrucous carcinoma (VC), and SCC.

There has not been compelling etiologic factors or risk factors identified for PVL. PVL affects both smokers and non-smokers.[6,61,64,73] A systematic review revealed that only 33.9% of patients had a form of tobacco habit in fourteen studies involving a total of 254 subjects.[63] Therefore, smoking or use of tobacco product is not strongly associated with PVL. Similarly, alcohol consumption also has not been associated with PVL with the prevalence of use ranging from 17% to 26%.[71,73,74]

Several studies have looked at infectious organisms as potential etiologic factors for PVL, but the results have been conflicting. Hansen et al.[6] and Silverman et al.[42,64] demonstrated the presence of *C. albicans* in some of the biopsy specimens of PVL, but Kahn et al.[75] did not observe *C. albicans* in their cohort. The relationship between HPV and PVL has also been inconsistent. Three studies have reported the presence of HPV in PVL, but two studies did not.[62,69,74,76,77] The pooled HPV positivity prevalence is 5% in PVL.[78] When Campisi et al.[74] compared the risk of HPV infection in PVL and in conventional OL, there was no significant difference between the two groups. Only one study has been conducted on the presence of

Epstein-Barr virus (EBV). Results showed that 60% of the patients with PVL exhibited EBV presence in the lesions.[79] Although infectious organisms are present in PVL lesions, studies have yet to explain the relationship between the infectious organisms and the pathogenesis of PVL.

In an attempt to understand the pathogenesis of PVL, over 20 tumor markers were examined by various investigators.[78,80] However, the majority of the studies were a single study and could not provide convincing proofs regarding their relationship to the pathogenesis of PVL.[78,80] Nevertheless, in two systematic reviews investigating markers of PVL, Rintala et al.[78] and Okoturo et al.[80] observed that DNA aneuploidy and mini chromosome maintenance protein (Mcm) may be potential markers for PVL.[71,72,75,78,80,81] However, further studies are still required to better understand these markers and to establish the pathogenesis of PVL.

The clinical importance of PVL is underscored by the high rate of malignant transformation, but definitive risk factors or tumor markers that may indicate transformation have not been established. The overall MTR of PVL is 52% with reports ranging from 17% to 100%.[63] In one study, the MTR was not different between smokers and nonsmokers with PVL.[64] DNA aneuploidy and Mcm expression have been correlated with the degree of dysplasia in PVL and may predict the malignant transformation.[71,72,75,81] However, more studies are necessary to validate these markers. There is some evidence that females patients with PVL may be at a higher risk for malignant transformation than males.[67,73] Gingiva is more frequently involved in carcinoma development when compared to non-PVL patients with OSCC.[64,73,82] Patients with PVL are more likely to develop VC than OSCC.[73] The overall mortality rate based on five descriptive studies with an average of 8.4 year follow-up is 30.1%.[63]

PVL has been treated with various methods including surgical excision, radiation, chemotherapy, and laser ablation; however, these methods often cannot prevent the progression

of PVL.[6,61,62,64,67,75] Thus far, surgery has been the most utilized form of treatment and is also preferred for the advantage of histopathological examination of the tissue.[83,84] Unfortunately, recurrence is common regardless of the treatment modality with the overall recurrence rate of 71.2%.[84] None of the treatment modalities have been able to effectively prevent the recurrence and progression of PVL.[83,84] Because of the refractory nature of PVL and the high rate of malignant transformation, diligent follow-up, frequent biopsy of suspicious or changing lesions, and prompt management of dysplastic lesions are recommended when treating patients with PVL.[66,84]

The diagnosis of PVL is difficult due to its retrospective nature, seemingly benign initial presentation and lack of standardized diagnostic criteria leading to delayed diagnosis. Temporal observation of lesions spreading and/or becoming multifocal is necessary for the diagnosis. There is no unique histological feature and early lesions are often benign showing hyperkeratosis without evidence of dysplasia. Therefore, only when the lesions progress to cover a large portion of the oral cavity with evidence of dysplastic changes or even development of malignancy would a clinician become concerned for PVL.

Furthermore, standardized diagnostic criteria have not been established at this time. Hansen et al.[6] provided ten histologic stages of PVL based on the clinicopathological features, which are listed in Table 1.2. However, Batsakis et al.[68] proposed four histological stages, removing the intermediate stages and the papillary squamous cell carcinoma (Table 1.3). Since then, two diagnostic criteria have been suggested by Cerero-Lapiedra et al.[85] and Carrard et al.[86] based on both clinical and histopathological presentations (Table 1.4 and 1.5). These criteria require the lesions to possess verrucous features clinically and/or histopathologically for the diagnosis of PVL. However, some authors suggest that verrucous appearance should not be

considered an essential aspect of the diagnostic criteria for PVL. Aguirre-Urizar states that by the time a lesion appear verrucous, often carcinoma is already present.[87] He also argues that proliferative and multifocality of the lesions are the most crucial aspect of PVL, hence proposing a new term "proliferative multifocal leukoplakia" to replace PVL.[87] In addition, Villa et al.[66] demonstrated that patients with PVL often had lesions with fissured and erythematous appearance, therefore, proposing the name PVL to be changed to "proliferative leukoplakia". The study also proposes a new diagnostic criteria (Table 1.6).[66] At this time, more discussions and studies are needed order to establish and confirm the effectiveness of standard diagnostic criteria for PVL that will allow early detection.

Table 1.1. Diagnostic criterial for oral epithelial dysplasia; adapted from El-Naggar et al.[7]

Architectural changes	Cytological changes
Irregular epithelial stratification	Abnormal variation in nuclear size
Loss of polarity of basal cells	Abnormal variation in nuclear shape
Drop-shaped rete ridges	Abnormal variation in cell size
Increased number of mitotic figures	Abnormal variation in cell shape
Abnormally superficial mitotic figures	Increased N:C ratio
Premature keratinization in single cells	Atypical mitotic figures
Keratin pearls within rete ridges	Increased number and size of nucleoli
Loss of epithelial cell cohesion	Hyperchromasia
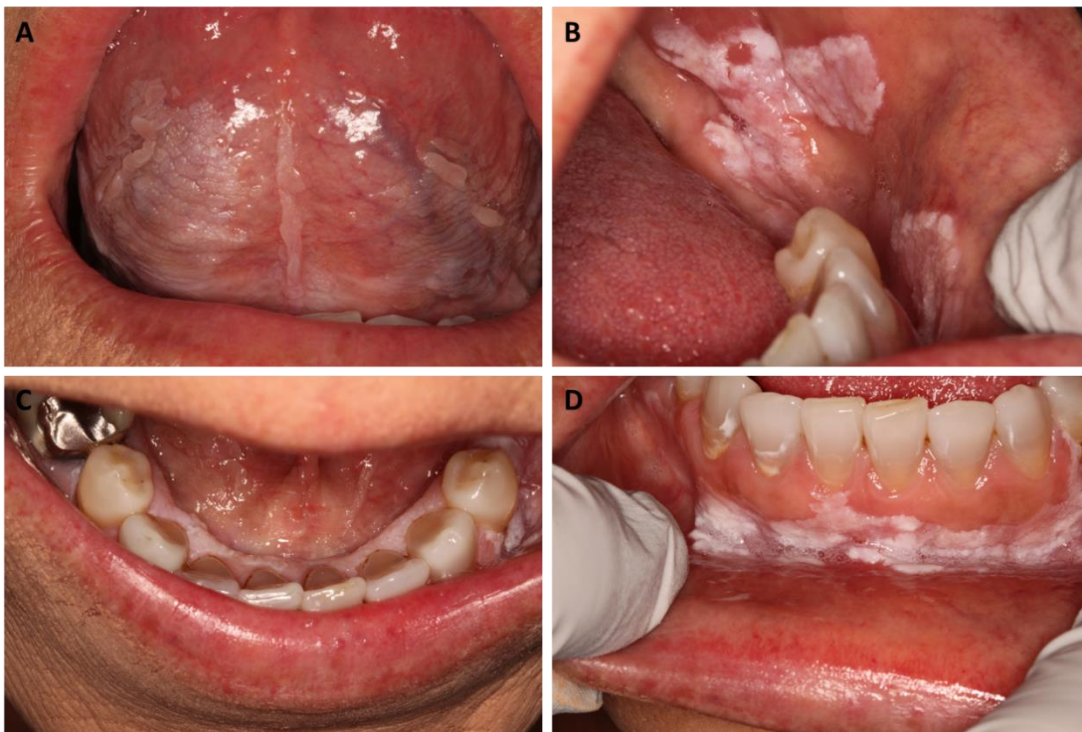
Figure 1.1. Clinical photos of proliferative verrucous leukoplakia. This patient has multiple leukoplakic plaques on oral cavity mucosa. **A**, Ill-defined leukoplakic plaque with fissured surface on ventral surface of the tongue. **B**. Thickened leukoplakic plaques on buccal mucosa, retromolar pad, and labial mucosa. Focal ulceration is evident on the buccal mucosal lesion. **C**. Leukoplakic lesion on the lingual gingiva of anterior mandible extending from premolar to premolar. **D**. Ill-defined leukoplakic plaque on facial attached gingiva and alveolar mucosa.

Table 1.2. Histologic stages of PVL by Hansen et al.[6]

Grade	Histology
0	Normal oral mucosa
1	
2	Homogeneous leukoplakia
3	
4	Verrucous hyperplasia
5	
6	Verrucous carcinoma
7	
8	Papillary Squamous carcinoma
9	
10	Less differentiated carcinoma

Table 1.3. Histologic stages of PVL proposed by Batsakis et al.[68]

Stage	Histology
1	Clinical flat leukoplakia without dysplasia
2	Verrucous hyperplasia
3	Verrucous carcinoma
4	Conventional squamous cell carcinoma

Table 1.4. Proposed diagnostic criteria for proliferative verrucous leukoplakia by Cerero-Lapiedra et al.; adapted from Cerero-Lapiedra et al.[85]

Major Criteria	Minor Criteria
A. A leukoplakia lesion with more than two different oral sites, which is most frequently found in the gingiva, alveolar processes and palate. B. The existence of a verrucous area. C. The lesions have spread or engrossed during development of the disease. D. There has been a recurrence in a previously treated area. E. Histopathologically, lesions range from simple epithelial hyperkeratosis to verrucous hyperplasia, verrucous carcinoma or oral squamous cell carcinoma, whether in situ or infiltrating.	a. An oral leukoplakia lesion that occupies at least 3 cm when adding all the affected areas. b. Female patient. c. Be a non-smoker (male or female). d. A disease evolution greater than 5 years

In order to be diagnosed as PVL, the patient should meet 3 major criteria (must include E) or 2 major criteria (must include E) and two minor criteria.[85]

Table 1.5. Proposed diagnostic criteria for proliferative verrucous leukoplakia by Carrard et al.; adapted from Carrard et al.[86]

1.	Leukoplakia showing the presence of verrucous or wartlike areas, involving more than two oral subsites. The following oral subsites are recognized: dorsum of the tongue (unilateral or bilateral), border of the tongue, cheek mucosa, alveolar mucosa or gingiva upper jaw, alveolar mucosa or gingiva lower jaw, hard and soft palate, floor of the mouth, upper lip and lower lip.
2.	When adding all involved sites, the minimum size should be at least 3 cm.
3.	A well-documented period of disease evolution of at least five years, being characterized by spreading and enlarging and the occurrence of one or more recurrences in a previously treated area.
4.	The availability of at least one biopsy in order to rule out the presence of a verrucous carcinoma or squamous cell carcinoma.

All four criteria should be met to be diagnosed as PVL.[86]

Table 1.6. Proposed diagnostic criteria for proliferative verrucous leukoplakia by Villa et al.[66]

1.	White/keratotic lesions that may be smooth, fissured, verrucous, or erythematous with or without ulcer.
2.	Multifocal non-contiguous lesions or a single large lesion >4.0 cm involving one site or a single large lesion >3 cm involving contiguous sites.
3.	Lesions that progress/expand in size and/or develop multifocality over time.
4.	Histopathology that, if not overtly exhibiting dysplasia or carcinoma, shows hyperkeratosis, parakeratosis, atrophy, or acanthosis with minimal to no cytologic atypia (KUS), with or without a lymphocytic band, or verrucous hyperplasia; these features must not support a diagnosis of frictional or reactive keratoses.

All four criteria must be met to be diagnosed as PVL.[66]

KUS, keratosis of unknown significance.

CHAPTER 2: DIFFERENTIAL EXPRESSION OF PROGRAM DEATH LIGAND 1 IN PROLIFERATIVE VERRUCOUS LEUKOPLAKIA

Introduction

In recent years, there has been a significant increase in immune therapy availability and research to treat various cancers.[88] One aspect of immune therapy is the regulation of T-cell activation in the cancer microenvironment. Blocking the inhibitory pathways of T-cells allows their activation and attack on cancer cells.[88,89] One of the inhibitory pathways has been identified as the program cell death 1 (PD-1)/program cell death ligand 1 (PD-L1) axis.[88,89] PD-1 is a surface receptor expressed on T-cells, B-cells, natural killer T-cells, activated monocytes, myeloid cells, and dendritic cells.[88,89] Its ligand, PD-L1, is a transmembrane protein expressed on T-cells, B-cells, dendritic cells, macrophages, endothelial cells, epithelial cells, mesenchymal stem cells, bone marrow-derived mast cells, and others.[88–90] Binding of PD-L1 to PD-1 triggers the inhibitory pathway of T-cell activation and downregulates cytotoxic activity.[88,89] In a normal tissue microenvironment, this pathway functions as an immune check point, allowing self-tolerance and regulation of T-cell response.[88] However, several cancers have been shown to upregulate the expression of PD-L1, inducing unwarranted inhibition of T-cell activation thus evading the immune surveillance.[88,89] During the past decade, several anti-PD-1 and anti-PD-L1 drugs have been developed to treat and manage different types of cancers (Table 2.1).[89] By blocking the PD-l/PD-L1 pathway, the drugs allow the cytotoxic T-cells in the cancer microenvironment to be activated and target the cancer cells.[88]

Currently, PD-L1 is studied extensively as a biomarker to predict treatment response with anti-PD-1/PD-L1 therapy, aiming to better stratify patients for the most appropriate therapy.[91] Several studies have demonstrated that expression of PD-L1 in certain cancer cells, detected most commonly using immunohistochemistry (IHC), have correlated with better response to anti-PD-1/PD-L1 therapy.[92–95] In fact, PD-L1 is utilized as a biomarker to determine the eligibility for treatment with certain anti-PD-1/PD-L1 drugs for some cancers.[91] For example, >50% PD-L1 expression in the tumor cells of non-small cell lung cancer (NSCLC) is required for the first-line treatment with pembrolizumab.[88,89] Unfortunately, PD-L1 has its limitations as a biomarker such as low negative predictive value.[88,91] Even with negative PD-L1 expression, some patients with malignant melanoma and NSCLC have shown to respond to anti-PD-1 therapy.[88] Therefore, it is unclear whether a patient will benefit from the drugs when there is lack of PD-L1 expression in the tumor cells. Consequently, other potential biomarkers are also under investigation that will allow better therapy response prediction.[91]

There are numerous reported and ongoing studies investigating the expression of PD-L1 in different types of cancer and its implications for treatment modality and prognosis. But there are only a few published reports of its participation in precancerous conditions such as oral epithelial dysplasia.[96–98] In this study, we are interested in the PD-L1 expression in lesions of proliferative verrucous leukoplakia (PVL), a precancerous condition with a high risk of malignant transformation into verrucous and/or squamous cell carcinoma.

In 1984, Silverman et al.[42] followed 257 patients with oral leukoplakia over a mean of 7 years to study the factors associated with malignant transformation. During the study, a subset of patients demonstrated unusual progression of the lesions, and these patients were further studied by Hansen et al.[6,42] According to Hansen et al.,[6] the lesions were initially benign and

diagnosed as hyperkeratosis histologically. Overtime, however, they became multifocal and/or

diffuse with dysplastic changes. Some became warty and verrucous in appearance and were

diagnosed as verrucous hyperplasia histologically. Eventually many of these lesions developed

into malignancies. These lesions were also found to be persistent and resistant to treatments such

as surgery and radiation, irrespective of the histological diagnosis. Because of the appearance

and proliferative nature of the leukoplakic lesions in this condition, Hansen et al.[6] coined the

term "proliferative verrucous leukoplakia (PVL)".

PVL most frequently affects females and occurs most often in the seventh decade of

life.[63] There have not been compelling etiologic or risk factors identified.[6,61,64,73]

Histopathologically, the lesions initially present as benign hyperkeratosis, but over time progress

through the spectrum of epithelial dysplasia as they spread and become multifocal.[61,62,64,96] In a

systematic review, Pentenero et al.[63] found that the overall rate of malignant transformation is

52%. Treatment modalities utilized thus far include surgical excision, radiation, laser ablation,

chemotherapy, photodynamic therapy, and topical medications, and all have been mostly

unsuccessful.[83,84] The overall recurrence rate is 71.2% regardless of the treatment modality.[84]

The clinical diagnosis of PVL is often missed or delayed due to its initial benign

presentation. Only when the lesions spread and progress towards malignancy without responding

to treatments is the clinician suspicious of the condition. There are no specific histological

features of PVL; therefore, clinical correlation is crucial. Although this is a rare condition, the

significance of the diagnosis is grave due to the high risk of malignant transformation and lack of

effective treatment modalities.

So far, no study has investigated PD-L1 expression in PVL patients. PD-L1 expression in

lesions of PVL may suggest anti-PD-1/PD-L1 therapy as a possible treatment option for PVL,

delaying or preventing the malignant transformation. Furthermore, PD-L1 may be utilized as a

biomarker to signal malignant transformation, which would be useful in monitoring the patients

and aiding in decisions regarding intervention and treatment initiation. The purpose of this study

was to explore the expression of PD-L1 in biopsy specimens of PVL lesions. We hypothesize

that lesions of PVL express PD-L1 and that there may be an association between the histological

stages of the lesions and the PD-L1 expression.

Table 2.1. Approved immunotherapy cancer drugs targeting PD-1/PD-L1 axis

Drug	Indication
Anti PD-L1	
Pembrolizumab (Keytruda®)	Metastatic non-small cell lung cancer Recurrent or metastatic head and neck squamous cell carcinoma Refractory or relapsed classical Hodgkin lymphoma Advanced cervical cancer Melanoma: advanced and adjuvant Advanced urothelial carcinoma Refractory or relapsed primary mediastinal large B-cell lymphoma Advanced hepatocellular carcinoma Advanced Merkel cell carcinoma Advanced gastric or gastroesophageal junction cancer MSI-H/dMMR cancers
Nivolumab (Opdivo®)	Metastatic non-small cell lung cancer Small cell lung cancer Advanced or metastatic urothelial carcinoma Melanoma Recurrent or metastatic head and neck squamous cell carcinoma MSI-H/dMMR metastatic colorectal cancer Renal cell carcinoma Hepatocellular carcinoma Classical Hodgkin lymphoma
Cemiplimab (Libtayo®)	Advanced cutaneous squamous cell carcinoma
Anti-PD-L1	
Atezolizumab (Tecentriq®)	Non-small cell lung cancer Urothelial carcinoma Triple-negative breast cancer
Avelumab (Bavencio®)	Metastatic Merkel cell carcinoma Advanced or metastatic urothelial carcinoma
Durvalumab (Imfinzi®)	Unresectable Stage III non-small cell lung cancer (NSCLC) Advanced or metastatic urothelial carcinoma

Methods and Materials

Patient Selection

The study design was a retrospective cohort study approved by the University of North Carolina Institutional Review Board. Biopsy specimens of oral lesions from patients with PVL that were submitted to the UNC School of Dentistry (SOD) Oral and Maxillofacial Pathology Service between July 1, 2007, and July 1, 2018 and diagnosed as hyperkeratosis, dysplasia, verrucous hyperplasia, or CIS were searched in the UNC SOD Electronic Patient Record. All specimens received by the service were submitted by clinicians providing oral and maxillofacial biopsies in the community and in the school of dentistry. From the identified patients, patients with a history of at least two biopsies of dysplasia from two different time points and/or at least two biopsies of dysplasia from two different sites were initially selected. Their hematoxylin-and-eosin (H&E) slides were retrieved and reviewed by a board certified oral and maxillofacial pathologist to confirm the diagnosis. The specimens were then categorized into two groups: a low-risk group which included hyperkeratosis and mild dysplasia, and a high-risk group which included moderate dysplasia, severe dysplasia and CIS. Verrucous hyperplasia was categorized based on the degree of dysplasia present. Patients with at least one low-risk group lesion and at least one high-risk group lesion and the corresponding specimens were identified and included in the study. The archived residual formalin-fixed and paraffin embedded (FFPE) tissue of the selected cases with sufficient remaining tissue were retrieved. The number of low-risk group specimens and high-risk group specimens were matched for each patient. For the control group, biopsy specimens of amalgam tattoo from non-PVL patients were identified and retrieved. The control specimens were randomly selected from the years that match the years the low-risk group lesion specimens were collected.

H&E

For each selected specimen, a new H&E slide was cut from the paraffin block. H&E stains of the FFPE tissue sections were carried out using an autostainer XL from Leica Biosystems (Buffalo Grove, IL). H&E stained slides were then digitally imaged in the Aperio ScanScope XT (Leica Biosystems, Buffalo Grove, IL) using a 20x objective. Each new H&E slide was examined by the board certified oral and maxillofacial pathologist to amend the diagnosis, if necessary, based on the new tissue section.

Immunohistochemistry

The expression of PD-L1 was evaluated using IHC. Rabbit monoclonal PD-L1 antibody clone CAL10 was obtained from Biocare Medical (#ACI3171A, Pacheco, CA)[97]. IHC of the FFPE tissue sections was carried out in the Bond fully-automated slide staining system (Leica Biosystems, Buffalo Grove, IL). Slides were dewaxed in Bond Dewax solution (AR9222, Buffalo Grove, IL) and hydrated in Bond Wash solution (AR9590, Buffalo Grove, IL). Heat induced antigen retrieval was performed for 20 min at 100ºC in Bond-Epitope Retrieval solution2 pH-9.0 (AR9640, Buffalo Grove, IL). The antigen retrieval was followed with 5 min Bond peroxide blocking (DS9800, Buffalo Grove, IL) and 10 min protein blocking (#BS966, Biocare Medical, Pacheco, CA) steps. After pretreatment, slides were incubated for 30 min with PD-L1 (1:100). Detection was performed using Bond Intense R Detection kit (DS9263, Buffalo Grove, IL) supplemented with the ImmPRESS HRP Anti-Rabbit IgG (Peroxidase) Polymer (#MP-7451-15, Vector Laboratories, Burlingame,CA). Stained slides were dehydrated and coverslipped. Tosillar tissue was employed as control tissue. Positive and negative controls (no

primary antibody) were included for each run. IHC stained slides were digitally imaged in the

Aperio ScanScope XT (Leica Biosystems, Buffalo Grove, IL) using 20x objective.

Scoring of PD-L1 Expression

For the evaluation of PD-L1 expression, three observers, an oral and maxillofacial

pathologist (RP), a dermatopathologist (PG), and the principal investigator (SL) were calibrated

using the tool called "PD-L1 Staining Interpretation Practice."[98] The evaluation was carried out

independently, and the order of viewing of the slides were randomized to minimize bias. When

necessary, the lesional areas of the tissue were defined prior to the evaluation, and the evaluation

was carried out only in the defined areas. The PD-L1 expression was defined as any viable

epithelial cells showing partial or complete membrane staining with any intensity.[99] Tissue

sections showing at least 100 viable epithelial cells were considered for evaluation.[99] The PD-L1

expression was then reported as an approximate percentage of cells stained; referred to as the

"score".[99] Based on the criteria for head and neck SCC, positive expression was defined as $\geq 1\%$

of the lesional cells expressing PD-L1, and negative expression was defined as <1% of the

lesional cells expressing PD-L1.[99] The cases where both $\geq 1\%$ and $< 1\%$ expressions were

reported by different observers, all observers reviewed the cases simultaneously and consensus

was reached. All observers utilized the digitally imaged slides using the Aperio ImageScope

(Leica Biosystems, Buffalo Grove, IL) slide viewing software.

Statistical Analysis

To assess agreement between the observers, we computed the intraclass correlation

coefficient (ICC) with observers as fixed. The median score was used to represent the

distributions of values. For testing, the scores were dichotomized into < 1% and ≥ 1% following the standard threshold for negative and positive PD-L1 expression, respectively, for head and neck SCC.[100] We further grouped the scores a-priori into finer-grained categories, 0, .5, 1-9, 10-50, and 51-100.

To test whether there was an association between the low-risk and high-risk groups and whether PD-L1 expressions were above the threshold, we conducted the Cochran-Mantel-Haenszel (CMH) test, with the patients constituting the strata. As a sensitivity analysis, we conducted another CMH test using the wider range of PD-L1 categories summarized in Table 2.5. An odds ratio (OR) was also calculated to further evaluate the association between the risk groups and PD-L1 expression. Our alpha was set a-priori at .05. All analyses were run in R (R core team, 2019, Vienna, Austria).

Results

Sixteen patients for the control group and 8 patients for the PVL cohort were selected for the study. Initially, 16 amalgam tattoo control specimens, 16 low-risk group specimens, and 16 high-risk group specimens were selected and stained for H&E and PD-L1 IHC. After reviewing the new H&E sections, 6 low-risk group lesions were re-classified as high-risk group lesions and 2 high-risk group lesions were re-classified as low-risk group lesions. One specimen was excluded due to insufficient tissue section after sectioning. Another specimen was excluded because it was later found to be a re-excision of another specimen included in the study. Therefore, 12 low-risk group lesions and 18 high-risk group lesions were available for analysis along with all 16 control specimens.

In the control group, there were 11 female and 5 male patients with an average age of 57 (range 20-84) at the time of biopsy. One patient was HIV positive. Medical and social histories of the remaining control patients were not available. Gingiva was the most common site of biopsy (8 out of 16). For patients with PVL, 7 patients were female, and 1 patient was male. The average age at the time of biopsy was 56 (range 46 to 73) for low-risk group lesions and 59 (range 49 to 73) for high-risk group lesions. Social histories of 6 patients were available. Two of the 6 patients were current smokers, both with a greater than 20 pack-year history. The remaining 4 out of the 6 patients have a distant history of smoking. Two patients reported consuming alcoholic beverages. Gingiva (9 out of 30) and tongue (8 out of 30) were the most common sites of biopsy for both low- and high-risk group lesions. The patient gender, smoking status, alcohol habit, and medical history information are presented in Table 2.2.

Table 2.3. summarizes the percent PD-L1 expression scored by each observer, the consensus values, histological diagnosis, age at biopsy, and biopsy site for each specimen. Statistically, interobserver agreement was very good (ICC=0.94, F(49,100)=48.9 , p<0.001). Median PD-L1 expression values are displayed in Figure 2.1 and summarized in Table 2.5. Median PD-L1 expression values were 0%, 0%, and 4% for control, low-risk group, and high-risk group, respectively. The PD-L1 expression categories are summarized in Table 2.6. All control specimens demonstrated PD-L1 expression of 0%, whereas only 35% of active lesions demonstrated no expression. Only 1 out of 12 (8.3%) low-risk group lesion displayed ≥ 1% PD-L1 expression in epithelium while 15 out of 18 (83%) high-risk group lesions displayed ≥ 1% PD-L1 expression in epithelium.

The CMH test of our hypothesis was significant (χ^2=8.3, df=1, p=.004), implying an association between the risk groups and PD-L1 expression. As a sensitivity analysis we reran the

test using our 5-level PD-L1 categories (Table 2.5). This analysis was also significant ($M^2=17$, df=8, p-value=0.03). The OR of high-risk lesions having PD-L1 expression ≥1% was 54, although no confidence interval could be estimated due to complete separation within the patient-level strata.

Table 2.2. Patient information for control and proliferative verrucous leukoplakia groups

Patient	Gender	Smoking/alcohol status	Medical history
Control			
1	Male	Unknown	Family history of oral cancer
2	Female	Unknown	
3	Female	Unknown	
4	Female	Unknown	
5	Female	Unknown	
6	Female	Unknown	
7	Female	Unknown	
8	Female	Unknown	
9	Male	Unknown	
10	Female	Unknown	
11	Male	Unknown	
12	Female	Unknown	
13	Male	Unknown	
14	Male	Unknown	
15	Female	Non-smoker	HIV +
16	Female	Unknown	
PVL			
1	Female	Unknown	Raynaud syndrome, possible LE, possible scleroderma
2	Female	>20-year history of smoking and drinking	
3	Female	50 pack-year history of smoking, quit >20 years ago	
4	Male	70 pack-year history of smoking, quit in 2001; alcohol on weekends (6 pack)	HTN, peripheral neuropathy
5	Female	5 pack-year history of smoking, quit in 1984; 1-2 drinks/week	Sjogren syndrome, Grave's disease, osteoarthritis, endometriosis
6	Female	>35 pack-year history of smoking	H/o breast cancer (2013), Barrett's esophagus
7	Female	Quit smoking >20 years ago	H/o of breast cancer, Hodgkin lymphoma, HCV; hypothyroidism, GERD, DM, HTN
8	Female	Unknown	

LE, lupus erythematosus; HTN, hypertension; H/o, history of; HCV, hepatitis C virus; GERD, gastroesophageal reflux disease; DM, diabetes mellitus.

Table 2.3. Summary of percent PD-L1 expression in epithelium, histology grade, age at biopsy, and biopsy site for each specimen

Patient	Specimen	PD-L1 Expression (%)			Consensus value	Histology	Age at biopsy	Biopsy site
		OB 1	OB 2	OB 3				
Control								
1	1	0	0	<1	0	AT	61	Gingiva
2	2	0	0	0	0	AT	64	FOM
3	3	0	<1	0	0	AT	30	Hard palate
4	4	0	0	0	0	AT	54	Gingiva
5	5	0	0	0	0	AT	58	Gingiva
6	6	0	<1	0	0	AT	62	Maxillary ridge
7	7	0	0	0	0	AT	66	Gingiva
8	8	0	0	<1	0	AT	20	Hard palate
9	9	0	0	0	0	AT	84	Gingiva
10	10	0	0	0	0	AT	66	"Under tongue"
11	11	0	0	0	0	AT	48	Gingiva
12	12	0	0	<1	0	AT	68	Gingiva
13	13	0	0	0	0	AT	50	FOM
14	14	0	0	<1	0	AT	69	Tongue
15	15	0	0	0	0	AT	54	Tongue
16	16	0	0	0	0	AT	59	Buccal mucosa
Low-Risk Group								
1	1	0	<1	<1	<1	LG	46	Lateral tongue
	2	0	0	0	0	HK	48	Gingiva
	3	0	0	0	0	LG	48	Gingiva
2	4	0	0	<1	0	LG	58	Tuberosity
	5	0	0	0	0	LG	58	Buccal mucosa
3	6	0	<1	0	0	HK	71	Maxilla
4	7	0	0	0	0	LG	68	Dorsal tongue
5	8	0	<1	0	0	LG	60	Gingiva
	9	0	<1	<1	<1	LG	62	Tuberosity
6	10	0	5	<1	0*	LG	49	Ventral tongue
	11	0	0	1	0*	LG	49	Soft palate
7	12	30	25	40	30	LG	53	Buccal mucosa

		High-Risk Group						
1	1	1	1	3	1	HG	50	Gingiva
	2	0	0	0	0	HG	52	Anterior palate
2	3	20	5	10	10	HG	58	Ventral Tongue
	4	0	2	2	1*	HG	58	Gingiva
3	5	0	15	20	3*	HG	70	Buccal mucosa
	6	0	5	3	3*	HG	73	Lateral tongue
	7	40	75	55	55	HG	73	Alveolar ridge/palate
4	8	3	7	25	5*	HG	63	Tongue
5	9	100	70	95	95	HG	62	Lateral tongue
	10	80	70	80	80	HG	62	Gingiva
6	11	0	3	0	0*	HG	49	FOM
	12	0	1	2	0*	HG	51	Buccal mucosa
7	13	100	100	100	100	HG	53	Lower lip
	14	0	1	2	2*	HG	47	Gingiva
	15	90	75	95	90	HG	55	Gingiva
8	16	5	15	25	15	HG	54	FOM
	17	2	7	5	5	HG	56	Lateral tongue
	18	0	5	5	1*	HG	56	FOM

PD-L1, program cell death ligand-1; AT, amalgam tattoo; LG, low-grade dysplasia; HG, high-grade dysplasia; FOM, floor of mouth.
*Consensus reached by all three observers by reviewing the slides simultaneously.

Table 2.4. Summary of average age and biopsy site by risk group

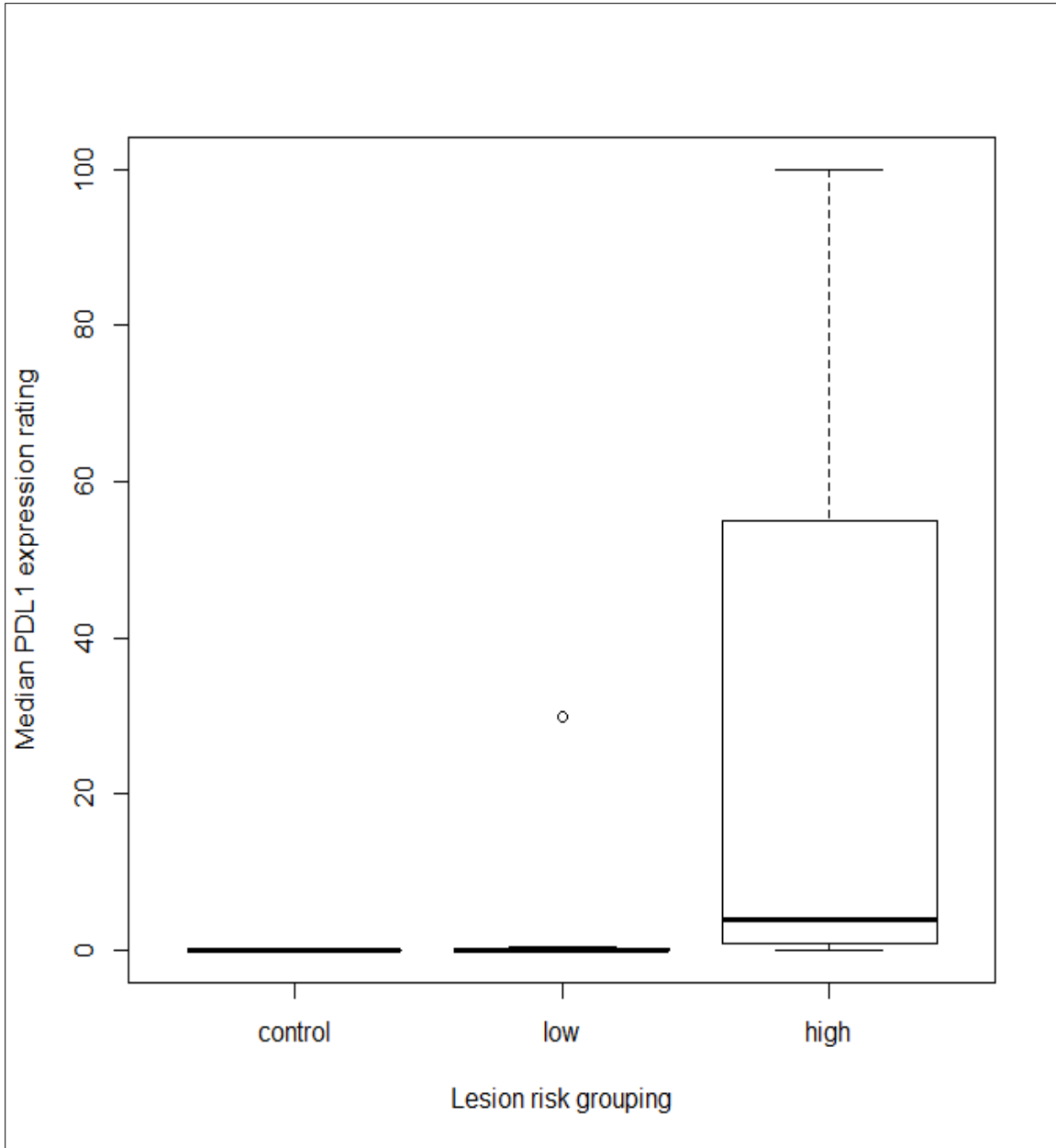|	Control	Low-Risk	High-Risk	
Average age at biopsy	57	56	59	
Biopsy site				**Total**
Gingiva	8	3	6	17
Tongue	2	3	5	10
Floor of Mouth	3		3	6
Hard Palate	2		2	4
Buccal mucosa	1	2	2	5
Tuberosity		2		2
Soft palate		1		1
Lower lip			1	1
Unknown		1		1

Figure 2.1. Boxplots of median percent PD-L1 expression in epithelium based on the risk group. The median PD-L1 expression for the control group (n=16) is 0%. The median PD-L1 expression for the low-risk group lesions (n=12) is 0%. The median percent PD-L1 expression for the high-risk group lesions (n=18) is 4%.

Table 2.5. Five-point summaries of PD-L1 consensus values

	Min	1st quartile	Median	Mean	3rd quartile	Max
Control (n=16)	0.0	0.0	0.0	0.0	0.0	0.0
Low-Risk Lesion (n=12)	0.0	0.0	0.0	2.6	0.1	30
High-Risk Lesions (n=13)	0.0	1.0	4.0	25.9	31.3	95

Table 2.6. Number of lesions with 0%, <1%, 1-9%, 10-50%, and 51-100% PD-L1 expression in epithelium

PD-L1 expression (%)	0	<1	1-9	10-50	51-100	≥ 1
Control (n=16)	16	0	0	0	0	0
Low-Risk Lesion (n=12)	9	2	0	1	0	1/12 (8.3%)
High-Risk Lesion (n=18)	3	0	8	2	5	15/18 (83.0%)

[a]Interobserver Agreement, ICC=0.94.
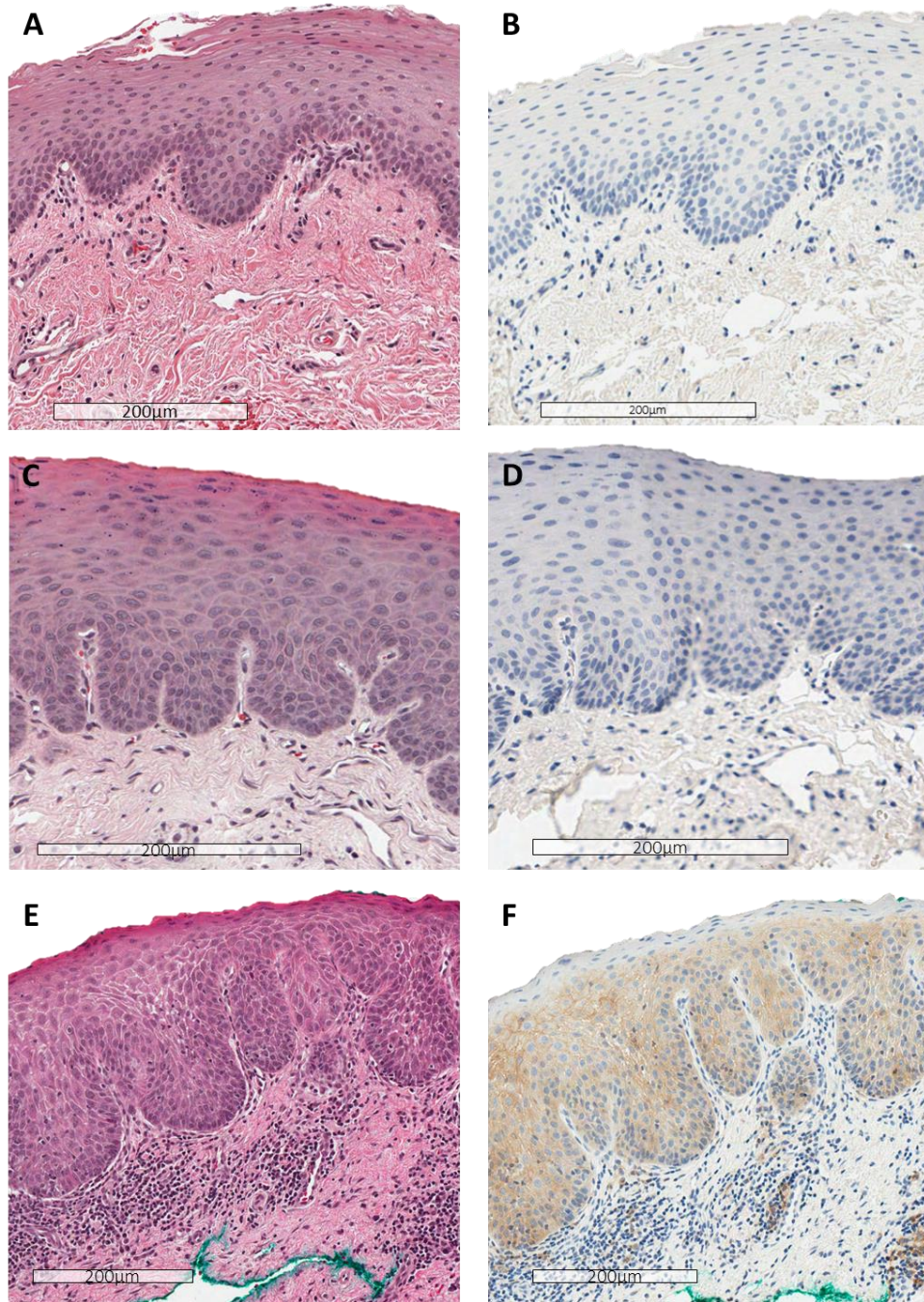
Figure 2.2. Representative photomicrographs of hematoxylin and eosin (H&E) and PD-L1 immunohistochemical (IHC) studies for control, low-risk group lesion and high-risk group lesion. **A**, H&E of control tissue (amalgam tattoo). **B**, PD-L1 IHC for the control tissue showing negative PD-L1 expression (0% expression). **C**. H&E of a low-risk group lesion (mild dysplasia). **D**, PD-L1 IHC for the low-risk group lesion (mild dysplasia), showing negative PD-L1 expression (0% expression). **E**, H&E of a high-risk group lesion (moderate dysplasia). **F**, PD-L1 IHC for the high-risk group lesion showing positive PD-L1 expression (95% expression).

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Discussion

Patient Demographics and Clinical Presentations

The demographics and clinical presentations of the PVL subjects included in this study reflect the features of PVL. The female-to-male ratio is 7:1, demonstrating the female predilection of PVL. This is similar to the ratio reported by Gouvêa et al.[71] but is much higher than the overall ratio of 2.5: 1 reported by Pentenero et al.[63] in a systematic analysis. The high female-to-male ratio of this study is likely due to the inclusion criteria that required both low-risk group lesions and high-risk group lesions, limiting the patient selection to those with more advanced PVL. There is some evidence that female PVL patients are at a higher risk for malignant transformation than male PVL patients.[67,73] This implies that PVL may progress more quickly in females, reaching the more advanced stages of PVL sooner. Consequently, the inclusion criteria of this study could have selected for females due to their possible tendency to present with more advanced disease, thus, explaining the much higher number.

One of the features of PVL is its progression towards malignancy over time which is reflected in the PVL cohort of this study. The average age at the time of biopsy for the high-risk group (59-years-old) was higher than the average age at the time of biopsy for the low-risk group (56-years-old). This correlates with the natural progression of PVL, which begins with a benign hyperkeratosis that, over time, spreads and progresses through the spectrum of dysplasia and then to malignancy. Therefore, patients are expected to develop a higher grade of dysplasia (high -risk group) later in life when the disease stage has advanced further.

Gingiva and tongue are the most commonly affected sites in this cohort, comprising 30% and 27% of the PVL specimens, respectively. This is also in agreement with the previously reported study, which found that gingiva, buccal mucosa, and tongue are the most common sites

affected by PVL.[63] The involvement of gingiva in PVL is a unique process. Although gingiva is

a commonly involved site for development of OL, malignant transformation is more common in

the tongue and floor of the mouth.[41–44] In PVL, however, gingiva is the site with the highest

malignant transformation[63], and our data seem to support this. The number of gingival

specimens in the low-risk group was 3 out of 12 (25%) while the number in high-risk group was

6 out of 18 (33%), implying that gingiva is more likely to develop a higher grade of dysplasia.

This suggests that the disease process leading to malignancy in PVL may differ from that of

other OPMDs. One should take caution, however, in establishing the relationship between the

gingival involvement and malignant transformation in our study due to the small sample size and

potential skewing of the samples based on the selection criteria.

Medical and social histories were available for some of the patients. Smoking history

was provided for 6 out of 8 patients with PVL. Only 2 of the 6 patients were current smokers,

both with a greater than 20 pack-year history. The remaining 4 out of the 6 patients had remote

histories of smoking, and most of them quit more than 20 years ago. Although smoking is not

considered an etiologic or risk factor for developing PVL, most of the patients in this study had

an extensive history of smoking. The temporal association between smoking and the onset of

PVL in this cohort is unknown, therefore, making any relationship between smoking and PVL is

difficult to assess.

Interestingly, 2 patients had autoimmune conditions (patient 1 and patient 5) reported by

the clinicians submitting the biopsies. The association between these conditions and PVL is

difficult to determine due to the small sample size. The conditions may have been the result of

advancing age, independent of PVL development, rather than related. In addition, 2 patients had

a history of breast cancer (patient 7 and patient 8), and one of them also had a history of Hodgkin

lymphoma. It is possible that these patients have unknown risk factors that may predispose them

to malignancies, including PVL, or the treatment of the malignancies may have contributed to

the development of PVL. Again, the association of their malignancy history and PVL cannot be

fully explored due to the small sample size and lack of access to the patients' full medical

histories.

Interobserver Agreement

For the expression of PD-L1 scoring, 3 observers independently assessed the samples.

One observer (OB 1) was a dermatopathologist with extensive experience in PD-L1 scoring

while the other two observers were newly trained for the purpose of the study. Despite the

differences in experience level, the agreement between the observers was very good (ICC=0.94).

This is partially due to a large number of specimens with an absence of PD-L1 expression. Of

46 specimens included in this study, 28 (61%) specimens displayed 0% PD-L1 expression in the

epithelium, and no expression of PD-L1 can be easily agreed upon among the observers.

Expression of PD-L1 in Normal Epithelium

All control specimens failed to demonstrate epithelial PD-L1 expression. This suggests

that normal oral cavity epithelium does not express PD-L1. Sieviläinen et al.[101] also reported

negative PD-L1 expression in 9 normal oral mucosal epithelium.[101] Similarly, Gonçalves et

al.[102] observed that normal oral mucosa (n=20) had absent to low PD-L1 expression in the

epithelium. It appears, however, that the latter study considered both membranous and

cytoplasmic staining as expression of PD-L1, whereas, our study limited the expression to

membranous staining only, as currently, the established practice for PD-L1 scoring is to assess

membranous staining and disregard the cytoplasmic staining.[97,99,100,102–104] This is rational since

PD-L1 is a transmembrane protein; therefore, it is functional when present on the cell surface.

Possibly, some of the low PD-L1 expression seen by the normal oral mucosa in the study by

Gonçalves et al.[102] would have been interpreted as negative if only the membranous stains had

been considered to be positive. A study with a larger sample size or a meta-analysis with a

standardized criterion for PD-L1 positivity should allow establishment of the appropriate level of

PD-L1 expression in normal oral mucosa. At this time, based on our results, along with the

limited available data, the normal oral mucosal epithelium appears to exhibit negative to low PD-

L1 expression.

Expression of PD-L1 in Abnormal Epithelium

Our study demonstrates expression of PD-L1 in the epithelium of some PVL lesions,

especially the high-risk group lesions. A recent study by Gonçalves et al.[102] reported that all oral

leukoplakia included in the study overexpressed PD-L1 in epithelium regardless of the degree of

dysplastic changes, which included no dysplasia, mild dysplasia, moderate dysplasia, and severe

dysplasia. Yagyuu et al.[105] also investigated PD-L1 expression in precancerous lesions and

found that some of the low-grade dysplasia (no dysplasia and mild dysplasia) and high-grade

dysplasia (moderate and severe dysplasia) expressed PD-L1 in the epithelium. Additionally,

they reported that increased PD-L1 expression was associated with increased risk for malignant

transformation and decreased rate of malignant-free survival.[105] Similarly, our study

demonstrated a significant number of high-risk lesions (15 out of 18) with ≥1% PD-L1

expression in the epithelium while only 1 out of 12 low-risk lesions showed ≥ 1% PD-L1

expression in the epithelium. The CMH test revealed that there is an association between the

risk groups and PD-L1 expression, and the odds ratio of high-risk lesions having PD-L1 expression ≥ 1% is 54.

Both Gonçalves et al.[102] and Yagyuu et al.[105] suggest the ability of the dysplastic epithelium to evade immunosurveillance by expressing PD-L1, disabling surrounding lymphocytes attempting to contain the disease. This may allow the dysplastic lesions to progress to higher grade of dysplasia and, eventually, to malignancy. In our study cohort, the clinical significance of this implication is the progression of PVL, and our data seem to support this idea. However, both Gonçalves et al.[102] and Yagyuu et al.[105] regarded cytoplasmic staining as expression of PD-L1; therefore, direct comparison to our study may not be entirely sensible. Furthermore, our results contradict the data presented by Sieviläinen et al.[101] who did not observe any PD-L1 expression in dysplastic epithelium of oral cavity.[101] Interestingly, they observed PD-L1 expression in the inflammatory cells in the lamina propria adjacent to dysplasia, which correlated positively with the degree of dysplasia. Unfortunately, the authors did not state the parameters used for the PD-L1 positivity in these inflammatory cells.

The presence of PD-L1 in some of the lesions of PVL, suggest its potential role as a biomarker for anti-PD-1/PD-L1 therapy. Currently, there is no effective treatment method for treating PVL, and yet the condition carries high rate of recurrence and high rate of malignant transformation.[63,83,84] It is possible that the current anti-PD-1/PD-L1 therapy utilized for various cancers may be beneficial for patients with PVL in either curing or containing the disease progression, especially in those who show PD-L1 expression and have lesions with higher degree of dysplasia.

Challenges Associated with the Study

Challenges associated with this study include small sample size, subjectivity in the grading of dysplasia, and lack of consensus concerning the diagnostic criteria for PVL. Our study included only 8 patients with PVL and a total of 30 PVL specimens. This is partially due to the rigorous inclusion criteria for the study to reduce the confounding factors by matching the number of low-risk and high-risk group lesions for each patient and to select for patients who truly present with PVL. PVL is also a rare condition; therefore, obtaining a large number of samples is difficult in a single institution. In order to improve the sample size, a multi-institutional study is desirable to provide a larger cohort and further assess the significance of PD-L1 expression in PVL lesions.

Currently, the WHO recommends the three-tier grading system for dysplasia: mild, moderate, and severe.[1] Problem with this grading system include poor reproducibility and significant interobserver variability.[7] Our tissues were graded by an experienced oral and maxillofacial pathologist. However, it is possible that some of these lesions would be graded differently by another pathologist, leading to a different study outcome. Various investigations have shown that a binary grading system improves agreement between observers.[15,16] We attempted to reduce the grading variability by essentially adopting the binary grading system. The specimens were grouped into a low-risk group, which included hyperkeratosis and mild dysplasia, and a high-risk group, which included moderate dysplasia and severe dysplasia, similar to the study method carried out by Yagyuu et al.[105]

There are several proposed diagnostic criteria for PVL, but lack of consensus. It is unclear which diagnostic criteria were utilized by the clinicians submitting the biopsy specimens included in our study as they were not specified in the requisition form. Our inclusion criteria

mitigated this issue by selecting patients with at least one lesion in each risk group. In addition, all of the selected patients had at least two biopsies of dysplasia from two different time points and/or at least two biopsies of dysplasia from two different sites. When the records of the patients included in the study were further reviewed, all patients met the criteria proposed by Cerero-Lapiedra et al.[85] which comprise the most strict diagnostic criteria proposed for PVL thus far.

Localization of the Expression of PD-L1 in PVL

Our study focused only on the expression of PD-L1 in epithelium of the lesions; however, PD-L1 expression in the adjacent inflammatory cells and its association with tumor-infiltrating lymphocytes (TIL) is an emerging prognostic and therapy predictive factor in some cancers.[91,106] A study by Kim et al.[106] found that in head and neck SCC, PD-L1 expression in the tumor infiltrating immune cells was associated with a better prognosis than the PD-L1 expression in the tumor cells. Of the three published studies that investigated PD-L1 expression in the stromal immune cells adjacent to dysplastic oral epithelium, only the study by Yagyuu et al.[105] provided patient outcome analysis.[101,102,105] They found that patients with higher number of PD-L1 positive subepithelial cells had worse 5-year malignant-free survival rate, which is in contrast to the data by Kim et al.[105] This raises the possibility that the immune responses differ in dysplasia compared to carcinoma. In summary, at this time, the data is too limited to determine the significance of PD-L1 expression in the immune cells associated with OED and its prognostic implication, thus requiring additional investigations.

Conclusion

The PD-L1 expression in various cancers has been a focus of cancer research in recent years, and the field is rapidly growing. Anti-PD-1 drugs such as pembrolizumab (Keytruda®) and nivolumab (Opdivo®) are currently being utilized for head and neck squamous carcinoma.[100,104] However, studies exploring PD-L1 expression in precancerous lesions and the utilization of anti-PD-L1/PD-1 drugs for these lesions have been scarce.

To our knowledge, this is the first study investigating PD-L1 expression in PVL lesions. we have demonstrated that PD-L1 is expressed in some PVL lesions, especially in high-risk lesions. Also, our data have shown an association between the degree of dysplasia and PD-L1 expression: higher the degree of dysplasia, more likely to express PD-L1. This implies that some patients with PVL may benefit from therapies that inhibit the PD-1/PD-L1 axis. Further studies with a larger cohort are necessary to better describe the PD-L1 expression in PVL and its potential role as biomarker for therapy.

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