A Retrospective 20-Year Analysis of Proliferative Verrucous Leukoplakia and Its Progression to Malignancy and Association with High-risk Human Papillomavirus

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Abstract

Proliferative verrucous leukoplakia (PVL) is defined as an aggressive, relentless and recalcitrant form of leukoplakia that has a high propensity for malignant transformation. The aim of this study was to evaluate the malignant potential of PVL and determine its possible association with high-risk human papillomavirus (HPV). Twenty cases with a clinical and biopsy proven diagnosis of PVL were collected from the University of Florida Oral Medicine clinic database. Immunohistochemistry was performed to evaluate the expression of p16^{INK4A} and p53 genes in the PVL lesions. The lesions were also tested for high-risk HPV by DNA in-situ hybridization. The average age of the patients at the time of first biopsy was 62.7 years. Most patients had multiple sites of involvement, gingiva being the most common location. The lesions progressed to malignancy in approximately 50% of patients. The expression of p16^{INK4A} gene was considered negative, with at least a 50–65% immunoreactivity observed in only three cases that progressed to malignancy. No expression of high-risk HPV was detected, whereas p53 staining was positive in less than 25% of the cells demonstrating gene expression. No definite association between PVL and high-risk HPV infection could be established. Due to the high transformation potential of PVL, early recognition with aggressive treatment, including multiple biopsies, and continued close clinical follow-up, remain the mainstay of favorable management of this condition.

Keywords Proliferative verrucous leukoplakia · Verrucous hyperplasia · Verrucous carcinoma · Squamous cell carcinoma · P16^{INK4A} · Human papillomavirus

Introduction

Proliferative verrucous leukoplakia (PVL) is an aggressive form of leukoplakia which has a higher propensity for malignant transformation [1], thus warranting special attention. PVL was first introduced into the literature by Hansen et al. in 1985 [1]. The World Health Organization (WHO) defined PVL as “a distinct and aggressive form of oral leukoplakia that potentially is a malignant disorder” exhibiting

high recurrence and malignant transformation rates [2]. Initially, it presents as a focal flat white lesion, which gradually spreads to become multifocal and diffuse. Over varying periods of time, the lesions become exophytic and wart-like and may transform to verrucous carcinoma (VC) or squamous cell carcinoma (SCC) [3]. PVL lesions tend to be persistent and are generally resistant to all treatment modalities, such as laser excision, surgery, radiation and chemotherapy. Hansen et al. described PVL as a ten stage histopathological continuum of disease which ranges from a simple hyperkeratosis, through verrucous hyperplasia (VH) to VC or SCC [1]. The disease may present in any of these evolutionary stages during its course. PVL related simple hyperkeratosis may thus be confused with conventional leukoplakia associated with trauma or other factors with minimal malignant potential. This is one of the reasons that PVL is often diagnosed late in its protracted course when the disease is in an advanced stage. In addition, the histopathological features of early stage lesions may be indistinguishable from non-PVL

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lesions of similar type. VH is a histologically defined entity, which was first described by Shear and Pindborg [4], and may come with varying degrees of dysplasia. Usually the lesions of PVL spectrum move up in grade and rarely regress [1]. Due to the high rate of field cancerization, PVL has a tendency to develop into malignant lesions at multiple sites in the oral cavity [5, 6]. Because of the lack of specific histologic criteria, its diagnosis is based on a combination of clinical and histopathologic findings. The wide histopathologic spectrum may complicate the early diagnosis of PVL, and create diagnostic and management challenges for both the clinician and oral pathologist. Early diagnosis, multiple sequential biopsies and long-term clinical follow-up, therefore, are critical for the adequate management of PVL in its premalignant stages.

The etiology of PVL remains unclear. On basis of the clinical and warty histological features of some PVL lesions, human papillomavirus (HPV) has been implicated in its pathogenesis [7]. Some authors have described an association of PVL with HPV [3, 8] whereas others were unable to confirm this association [9, 10]. The objectives of this study were to evaluate the malignant potential of PVL in patients seen at the University of Florida Oral Medicine Clinic, and determine its association with tumor suppressor gene p16^{INK4A}, a surrogate marker for transcriptionally active high-risk HPV. The lesions that showed at least focal reactivity to p16^{INK4A} were analyzed further for the expression of high risk HPV 16/18 by in-situ hybridization (ISH). High-risk HPV infection can lead to malignant transformation by inactivation of the p53 gene [11]. Assuming that the presence of p53 alteration coupled with HPV infection may have a role in the pathogenesis of PVL, and considering previous reports of aberrant p53 expression in PVL [12–14], we additionally evaluated the expression of p53 gene in the lesions that were subjected to high-risk HPV ISH testing.

Materials and Methods

Study Design and Data Collection

After approval from Institutional Review Board (IRB), a retrospective search for PVL patients, seen from 1994 to 2016, was performed in the UF College of Dentistry Oral Medicine Clinic Database. The inclusion criteria included; (a) patients with a clinical diagnosis of PVL, which required multifocal and diffuse involvement, and (b) patients with a biopsy proven lesion supportive of the clinical diagnosis of PVL. The diagnosis was based on a combination of clinical and histopathologic findings. Exclusion criteria included cases with one or more excisional biopsy but either no follow-up or a follow-up of less than 3 years, unless malignant transformation occurred before that time. We were able to select 20

PVL cases that met these criteria. Patient demographics with respect to age, sex, and location of lesions were recorded for each patient. The risk factors collected were history of tobacco use (yes or no), previous or existing medical conditions, prior malignancy, and p16^{INK4A}, p53 and HPV status. The clinical course of PVL was investigated by determining the average number of biopsies performed per patient, type of malignant transformation (VC or SCC), and the recurrence rate. The treatment methods (surgical excision, laser ablation, radiation), histological diagnoses, and treatment outcomes were recorded. Lesions were scored according to the severity on a scale of 1–10, according to the Hansen et al. criteria [1]. Briefly, grade 0 represented normal oral mucosa, grade 2 comprised of a simple hyperkeratosis with little or no dysplasia, grade 4 represented VH exhibiting little or no dysplasia, grade 6 comprised of VC, grade 8 lesion represented a papillary SCC, and grade 10 lesion was representative of moderate or poorly differentiated SCC. Any intermediate lesion was assigned a grade with odd numbers. Follow-up time for each patient was recorded in years. All patients were periodically followed in our clinic until either lost to follow-up or death. Hematoxylin and eosin (H&E) stained slides of all biopsies for each patient were retrieved. These were reviewed and examined by two board-certified oral pathologists (SF and DC).

Immunohistochemical Analysis and HPV In-Situ Hybridization

Formalin-fixed, paraffin-embedded (FFPE) tissue sections of 4 µm thickness were prepared and mounted on glass slides. Each section was coded and subjected to a standard immunohistochemical (IHC) staining protocol. For each patient, p16^{INK4A} IHC staining was performed on benign and pre-malignant lesions occurring at the same and/or different sites, and on malignant lesions in cases where patient developed a carcinoma. FFPE tissue sections were subsequently stained using a Benchmark Autostainer (Ventana Medical Systems, Tucson, Arizona). Endogenous peroxidase activity was quenched by incubating the slides in peroxidase blocking reagent for 10 min. Incubation with mouse anti-human p16^{INK4A} monoclonal antibody (dilution 1:100, Santa Cruz Biotechnology, Dallas, TX) or anti-p53 antibody (dilution 1:100, Dako, Carpinteria, CA) was performed for 30 min at room temperature. Sites of binding were detected using Optiview 3,3'-diaminobenzidine (DAB) as chromogen, according to the manufacturer's instructions. The sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. For ISH, 4 µm thick sections were prepared from FFPE tissue. DNA ISH for HPV16 and HPV18 was performed using the GenPoint Tyramide Signal Amplification System (Dako, Carpinteria, CA) according to the

manufacturer's protocol. Adequate positive and negative controls were used for IHC and ISH.

Based on previous published criteria, both nuclear and cytoplasmic reactivity of > 75% was considered positive for p16^{INK4A} [15]. Any staining less than 75% was interpreted as negative and graded on the following scale: score 0, frankly negative; 1–25%, score 1; 26–50%, score 2; 51–75%, score 3. PVL lesions that exhibited focal reactivity to p16^{INK4A} were evaluated for p53 and HPV16/18 expression by IHC and ISH respectively. Normal oral mucosa is expected to demonstrate minimal to no p53 gene expression [14]. Positive p53 staining was scored as follows: < 25% tumor cells stained (score 1); 25–50% (score 2); 51–75% (score 3); and > 75% tumor cells stained (score 4). For HPV ISH, nuclear staining was the criteria for positive expression, and the lesions were classified as either positive or negative.

Statistical Analysis

Data were analyzed using IBM SPSS version 24.0 statistical software (Chicago, IL, US). The Chi square test was used to assess statistical differences or association among categorical variables. Fisher's Exact test was used whenever the sample size was small. p-values of ≤ 0.05 were considered statistically significant.

Results

The clinical and histopathological findings of the 20 patients are compiled in Table 1. The average age at the time of first biopsy was 62.7 years, with an age range of 34–87 years. Six of the 20 patients were male (30%), with an average age of 66.8 years (range 57–87 years) at the time of initial biopsy. Fourteen patients were female (70%), with an average age of 61 years (range 34–75 years), resulting in a female to male ratio of 2.3:1. At the time of diagnosis, 60% patients reported a previous or current history of tobacco use; 25% were non-smokers and the history was unavailable for the remaining 15% patients. Of the tobacco users, 25% had quit smoking at the time of diagnosis. The previous or existing medical conditions included diabetes mellitus (n = 6), cardiac problems like myocardial infarction and heart murmurs (n = 2), anemia (n = 2), bisphosphonate use for osteopenia (n = 1), rheumatoid arthritis (n = 1), asthma (n = 1), SCC of the skin (n = 2), and a prior VC of the mandible (n = 1).

Leukoplakia was present on two or more intraoral sites in the majority of patients. Gingiva was the most common location (85%), followed by the palate (45%), tongue (35%), buccal and alveolar mucosa (25% each), and the floor of mouth (10%, Table 1). Lesions of the gingiva were either bilateral or involved all four quadrants in the majority of patients. The histological patterns observed for benign and

premalignant lesions were hyperkeratosis (HK), HK with chronic lichenoid mucositis (CLM, Fig. 1b), HK with dysplasia (Fig. 1d), VH (Fig. 1f), VH with CLM, and VH with dysplasia.

An average of 4.3 biopsies (range 1–15 biopsies) were performed for each patient during the follow-up period. At the time of first biopsy, 12 lesions had a grade 2 histologic diagnosis, equivalent to simple HK with little or no dysplasia (Table 1). Three patients had grade 4 lesions representing VH with little or no dysplasia at the time of first biopsy, and one had a grade 5 lesion. The lesions gradually progressed in all patients ranging in severity from grade 3–10. In 6 patients, the lesions had histologic features of VC (grade 6), one progressed to papillary SCC (grade 8), and 2 patients developed moderate to poorly differentiated SCC (grade 10, Table 1).

In an average follow-up time of 7.65 years (range 1–18 years) from the time of diagnosis or first biopsy, 9 of the 20 patients (45%) developed malignancy. The average age of patients in the cancer progression group and non-progression group was 66.44 years (range 46–87 years) and 59.73 years (range 34–73 years) respectively. No statistical difference was found between ages of the two groups. Women were more likely to develop malignancy (88.9%) than men (11.1%). Of the patients who developed cancer, four were ex-smokers and four non-smokers, and the history of tobacco use was unreported for 1 patient. In 6 patients, one site progressed to malignancy, whereas two or more sites were involved in 3 patients (Table 1). The most common site of malignant transformation was the gingiva (average time, 6.5 years), followed by the tongue, buccal mucosa, alveolar mucosa, and palate (Table 1).

PVL was treated by continued observation, surgical excision, or laser ablation (Table 1). The malignant lesions were surgically excised with wide margins. Three patients with initial PVL-associated carcinoma developed a second malignancy at another site (Table 1). These patients were treated by hemimaxillectomy or partial mandibulectomy, with or without neck dissection (Fig. 2a–d). One patient received radiation treatment but was later lost to follow-up. All patients developed persistent PVL lesions in the same locations with few patients developing additional lesions at new sites (Table 1).

p16^{INK4A} IHC was performed on select sequential biopsy specimens of each patient from the same site and from different sites, if applicable. The p16^{INK4A} expression was considered negative as none of the lesions met the > 75% nuclear and cytoplasmic staining criteria. The lesions were frankly negative (score 0) for p16^{INK4A} expression in 9 patients, whereas the remaining showed a weak patchy or diffuse staining which ranged from a score 1–3 (Table 2; Fig. 3a, c, e, see “Materials and Methods” for grading criteria). The majority of the p16^{INK4A} negative lesions, that exhibited

Table 1 Clinical and histologic findings in 20 patients with PVL

Case	Age ^a (years)	Sex	Tobacco use	Medical conditions/ prior malignancy	No. of biopsies	Location	Histologic grade of lesion ^b Least most	Transformation site	FU (years)	Outcome	Treatment
1	57	M	Yes	None	5	RLAM, G, HP LBM	2 4	None	13	Persistent lesions (same and new sites)	Laser ablation
2	87	M	NA	DM, MI, anemia	3	HP, LLAM	2 8	HP	1	SCC (same site), with persistent lesions	Surgical excision
3	62	F	Yes	DM, anemia	4	LT; RBM, LBM, G, VT, DT	2 6	T	7	VC one site persis- tent lesions (same sites)	Surgical excision and laser ablation
4	47	F	NA	None	2	G	3 4	None	9	Persistent lesions at same site	Surgery and gingivec- tomy
5	74	F	No	DM, BPs for osteo- penia, asthma, heart murmur	4	G	2 6	G	15	VC (same site)	Laser ablation
6	63	M	Yes	Multiple SCC of skin	2	LSP, G	2 4	None	7	Persistent lesions at same sites	–
7	34	F	Yes	None	3	G	3 4	None	5	Persistent lesions (same and new sites)	Surgical excision
8	57	F	Yes	VC R. Mand	15	G, P	2 10	G	15	Multiple malignan- cies (same and dif- ferent sites), with persistent lesions at same sites	Multiple laser abla- tions, hemimaxil- lectomy, partial mandibulectomy, neck dissection
9	61	F	Yes	None	7	G, P, BM, T	4 6	G	7.5	VC (same site) and persistent lesions (same and new sites)	Laser and wide surgi- cal excision
10	62	F	No	None	5	HP, T, G	2 4	None	9	Persistent lesions (same and new sites)	Surgical excision
11	46	F	Yes	RA	5	G, SP, UAM, VT, FOM, BM	4 10	LUAM	4	SCC (same site), with persistent lesions at same sites	Laser, hemimaxil- lectomy, neck dissection, radiation therapy
12	73	F	Yes	None	3	G	4 4	None	5.5	Persistent lesions (same and new sites)	Surgical excision
13	67	F	NA	SCC skin	2	G	2 3	None	7	Persistent lesions at same site	Surgical excision

Table 1 (continued)

Case	Age ^a (years)	Sex	Tobacco use	Medical conditions/ prior malignancy	No. of biopsies	Location	Histologic grade of lesion ^b Least most	Transformation site	FU (years)	Outcome	Treatment
14	60	F	Yes	None	2	G, SP	3 4	None	4.5	Persistent lesions same sites	Surgical excision
15	73	F	No	DM	7	G, LBM, T	2 6	LBM, T	6.5	VC, 2 sites (same and different)	Surgical excision and laser
16	63	F	No	None	3	G, LLAM	5 6	RG, LG	5	VC, 2 sites (same and different)	Marginal mandibulectomy, vestibuloplasty, laser
17	64	M	Yes	None	2	G	2 4	None	4	Persistent lesions at same site	Surgical excision
18	68	M	Yes	DM	4	VT, LLAM, FOM	3 7	FOM	3	CIS one site, with persistent lesions at same sites	Surgical excision and laser
19	75	F	No	DM	7	G	2 6	G	18	VC two times (same site)	Wide surgical excision
20	62	M	Yes	None	1	HP, T	2 -	None	7	Persistent lesions at same sites	Surgical excision
Mean	62.7	-	-	-	4.3	-	-	-	7.65	-	-

BM buccal mucosa, *BPs* bisphosphonates, *DM* diabetes mellitus, *DT* dorsal tongue, *FOM* floor of mouth, *FU* follow-up, *G* gingiva, *HP* hard palate, *L* left, *LAM* lower alveolar mucosa, *MI* myocardial infarction, *P* palate, *R* right, *RA* rheumatoid arthritis, *SCC* squamous cell carcinoma, *SP* soft palate, *T* tongue, *UAM* upper alveolar mucosa, *VC* verrucous carcinoma, *VT* ventral tongue, *Yrs* years

^aAge at first biopsy

^bLesions were scored according to the severity on a scale of 1–10 according to the scheme of Hansen et al. Any grade of 7 or above is equivalent to a SCC. Lesions that could not be definitively graded with even numbers according to the above criteria were assigned an intermediate grade, designated by an odd number from 1 to 9. For example, grade 3: Severe hyperkeratosis, hyperkeratosis with dysplasia, or chronic ulcerative stomatitis (CUS); grade 5: severe verrucous hyperplasia; grade 7: Carcinoma-in-situ

Fig. 1 Representative benign and premalignant lesions of PVL spectrum (**a**). Thin, homogenous leukoplakia in case #15, representative of the histologic diagnosis of hyperkeratosis and chronic lichenoid mucositis [(**b**, H&E 10×), **c**]. Thick leukoplakia in case #12 repre-

senting severe hyperkeratosis with mild epithelial dysplasia [(**d**, H&E 10×), **e**]. Thicker, verrucoid and fissured leukoplakia around gingival margins of maxillary anterior teeth in case #11, depicting verrucous hyperplasia, and areas of keratin clefting (**f**, H&E 10×)

some focal reactivity, either retained or gained p16^{INK4A} protein expression over time (Table 2). Three lesions that progressed to malignancy gradually gained expression over time (cases #5, 15 and 19, Table 2). Two patients with biopsy proven diagnosis of chronic ulcerative stomatitis (CUS), who later developed VH and VC, gained p16^{INK4A} expression over a time of 7 and 3 years, respectively (Tables 1, 2). However, in our study cohort we did not find any significant association between malignant transformation and p16^{INK4A} immunoreactivity (chi square, $p=0.658$). IHC staining for p53 protein expression demonstrated less than 25% (score 1) nuclear staining in the lesional tissues (Fig. 3b, d, f). This staining was restricted to the basal and intermediate supra-basal layers of the epithelium in most lesions. Expression of HPV types 16 and 18 was not detected in any of the PVL lesions tested by DNA ISH.

Discussion

A definitive diagnosis of PVL is often difficult because of the various evolutionary stages in its broad histopathologic spectrum, clinical variations, and inadequate or maloriented biopsy specimens. And confusion with trauma-induced HK confounding this may lead to inadequate treatment of the condition. PVL studies published in the past followed the

diagnostic criterion developed by Hansen et al. [1]. Later, a reformulated criteria for the early diagnosis and facilitated management of PVL, which included five major and four minor criteria, was recommended by Cerero-Lapiedra et al. [16]. Agreeing with Bagan et al. that these criteria may be too complex for those who do not have significant clinical experience with PVL, Cerrard et al. simplified the list of diagnostic criteria [17].

The VH component of PVL is open to multiple interpretations and leads to some confusion. Shear and Pindborg regarded VH as a distinct clinicopathologic entity which may clinically and histologically resemble VC [4]. Over a period of time, it may evolve into VC or SCC [4]. Batsakis et al. and Slootweg et al. considered VH as an early stage and a morphologic variant of VC respectively [18, 19]. Murrar et al. considered that VH has the same biologic potential as VC, and recommended that it should be treated like a VC [20]. However, VH is not exclusive to PVL or the oral cavity [20]. In other head and neck mucosal sites, such as the sinonasal tract and larynx, VH may arise de novo or be associated with papillomas or oral florid papillomatosis. Thus, VH of the PVL spectrum may be misleading to the pathologists and clinicians.

Our study reported a malignant transformation rate of 45% in PVL lesions. There was a little difference in the average follow-up time between the 9 patients who developed

Fig. 2 Representative malignant lesions of PVL spectrum, **a** Verrucous carcinoma arising in anterior mandibular gingiva in case #16, **b** Verrucous carcinoma displaying broad pushing borders, keratin clefting, hyperkeratosis and a stromal inflammatory response (H&E, 10×), **c** Clinical representation of squamous cell carcinoma on right

mandibular gingiva in case #8. **d** Squamous cell carcinoma showing invasion in the deeper stroma in the form of clusters and islands of neoplastic cells. Squamous eddies and keratin pearls are also seen (H&E, 10×)

carcinomas and the patients in the non-progression group (8.7 and 7.6 years respectively). In the past, tobacco use has not been strongly associated with PVL [2]. However, role of tobacco in PVL lesions remains unclear since these lesions are seen in both smokers and non-smokers, and smoking histories are highly variable [1, 9, 21–23]. In our study, 60% patients had a history of tobacco use. In Hansen et al. study, 62% patients were tobacco users [1]. We speculate that, unlike previous reports, demographic and geographic variations probably reflecting differences in oral habits might be responsible for this discrepancy in tobacco usage seen in our patient cohort. Of the 9 lesions that transformed to malignancy, 7 patients had previous or current medical conditions. In the cancer non-progression group, 8 of the 11 patients had no existing medical risk factors, whereas 2 had previous SCC of the skin and one suffered from diabetes (Table 1). This may suggest a potential role of previous or existing medical conditions in the malignant transformation of PVL. However, larger cohort studies are needed to evaluate this

risk factor. In patients with one site of transformation, the malignant lesions developed in the site of PVL lesion. In patients with multiple malignancies, the same and additional sites of involvement were affected.

A strong overexpression of p16^{INK4A} is seen in HPV-related tumors [24]. In HPV-dependent lesions, function of retinoblastoma (pRB) is inactivated as a consequence of the expression of HPV-E7 protein [25]. In head and neck, HPV-induced cancers have been consistently shown to have fewer genomic mutations than HPV-negative malignancies, specifically HPV-associated cancers are less likely to have mutated p53 gene [26]. High-risk HPV expression and a p16^{INK4A} expression greater than 75% were not detected in the PVL spectrum lesions analyzed in our study (Fig. 3a, c, e). Few studies that performed IHC to determine p16^{INK4A} expression in PVL lesions did not specify a cutoff limit for interpretation of the positive staining [27, 28]. Importantly, previous studies that evaluated the expression of HPV in PVL utilized polymerase chain reaction (PCR)-based DNA

Table 2 Immunohistochemical and in-situ hybridization profile for p16^{INK4A}, p53 and HPV respectively

Case	Location	Biopsies	Time between biopsies (y)	Histologic diagnosis	p16 ^{INK4A}	p53	HPV
1	Hard palate	1st	4	VH + LM	Score 0	NP	NP
		2nd		VH + atypia	Score 0		
2	Anterior palate	1st	1	Severe VH	Score 0	NP	NP
		2nd		SCC	Score 0		
3	L mand mucosa	1st	5	HK	Score 0	Score 1	-ve
		1st		HK + MD	Score 0		
4	Tongue	1st	5	VC	Score 0	NP	NP
		2nd		VH	Score 0		
5	Buccal mucosa	1st	7	CLM/CUS	Score 0	NP	NP
		1st		VH	Score 1		
6	Gingiva	1st	–	HK + atypia	Score 1	NP	NP
		2nd		VH + MD	Score 0		
7	Gingiva	1st	3 ^{1/2}	VC	Score 3	Score 1	-ve
		2nd		VH + CLM	Score 1		
8	Gingiva	1st	2	VH	Score 2	Score 1	-ve
		2nd		VH	Score 0		
9	Gingiva	1st	3 ^{1/2}	VH	Score 0	NP	NP
		2nd		VH	Score 0		
10	Man. Gingiva	1st	5	VH + MD	Score 0	NP	NP
		2nd		SCC	Score 0		
11	R Max Gingiva	1st	5	VH + CLM	Score 0	NP	NP
		2nd		SCC	Score 0		
12	L Max Gingiva	1st	–	HK + CLM	Score 0	NP	NP
		1st		Severe VH	Score 0		
13	L Mand Gingiva	1st	7	VC	Score 0	NP	NP
		2nd		VH	Score 0		
14	R Max gingiva	1st	1	Severe VH	Score 0	NP	NP
		2nd		HK	Score 0		
15	L Max gingiva	1st	6	VH + CLM	Score 1	Score 1	-ve
		2nd		SCC	Score 0		
16	L Max gingiva	1st	4	VH + CLM	Score 0	NP	NP
		2nd		SCC	Score 0		
17	Hard palate	1st	2	VH + HK	Score 0	NP	NP
		2nd		VH + MD	Score 0		
18	L Mand gingiva	1st	8 mo	VH + CLM	Score 0	Score 1	-ve
		2nd		HK + CLM	Score 1		
19	R Mand gingiva	1st	1	HK	Score 1	NP	NP
		2nd		Severe HK	Score 1		
20	R Max gingiva	1st	1 mo	Severe HK	Score 2	NP	NP
		1st		VH	Score 2		
21	L Max gingiva	1st	3	CLM/CUS	Score 0	NP	NP
		2nd		VC	Score 2		
22	L Mand gingiva	1st	2	HK + CLM	Score 3	Score 1	-ve
		2nd		HK	Score 0		
23	L Mand gingiva	1st	4	Severe VH	Score 0	NP	NP
		2nd		VC	Score 0		
24	R Max gingiva	1st	4	VH	Score 0	NP	NP
		2nd		VH + LM	Score 1		
25	L Max gingiva	1st		HK + LM	Score 1	NP	NP
		1st		VH	Score 0		
26	L Mand Alv ridge	1st	2	VH	Score 0	NP	NP
		2nd		VH	Score 1		
27	L Max gingiva	1st	–	HK	Score 0	NP	NP
		2nd		VH	Score 1		
28	L Max gingiva	1st	5	VC	Score 2	Score 1	-ve
		3rd		VH + CLM	Score 0		
29	Hard palate	1st	–	VH + CLM	Score 0	NP	NP

Table 2 (continued)

CLM chronic lichenoid mucositis, *CUS* chronic ulcerative stomatitis, *HK* hyperkeratosis, *L* left, *LM* lichenoid mucositis, *MD* mild dysplasia, *mo* months, *NP* not performed, *R* right, *SCC*, squamous cell carcinoma, *VH* verrucous hyperplasia, *VC* verrucous carcinoma, *y* years, *-ve* negative

Fig. 3 Representative images of p16^{INK4A} and p53 protein expression (**a, b**). p16^{INK4A} and p53 IHC staining each representative of score 1 in case #13 (IHC, 20×) (**c, d**). p16^{INK4A} and p53 protein expression representative of score 2 (showing patchy nuclear and/or cytoplasmic staining) and score 1 respectively in case #5 (IHC, 20×) (**e, f**).

p16^{INK4A} and p53 protein expression representative of score 3 (showing diffuse nuclear and cytoplasmic staining) and score 1 respectively in case #15 (IHC, 20×) (**i, j**).

Table 3 Evaluation of HPV and p53 expression in PVL

Case	HPV+/total cases	HPV types	Detection technique	p53	Detection technique	Reference
1	8/9	HPV 16 (n=7) HPV 18 (n=1)	PCR	NP	NP	Palefsky et al. [3]
2	2/10	HPV 16 (n=1) HPV 18 (n=1)	PCR	8/10	IHC, PCR	Gopalakrishnan et al. [14]
3	0/10	NA	PCR	4/10	IHC	Fettig et al. [9]
4	50/50	HPV 11 HPV 16 HPV18	PCR	NP	NP	Femiano et al. [29]
5	14/58	HPV 16 (n=4) HPV 18 (n=10)	PCR	NP	NP	Campisi et al. [8]
6	0/13	HPV-6, 11, 16,18, 26, 31, 33, 35, 40, 45, 51, 52, 56, 59	PCR	NP	NP	Bagan et al. [6]
7	0/13 tested (n=20)	HPV 16 HPV 18	ISH	13/13 tested (n=20)	IHC	Our study

HPV human papillomavirus, *IHC* immunohistochemistry, *ISH* in-situ hybridization, *NA* not available, *NP* not performed, *PCR* polymerase chain reaction

detection techniques in contrast to ISH performed in our study (Table 3) [3, 8–10, 14]. Therefore, due to differences in investigation techniques and interpretation criteria, it is

difficult to compare results from the various studies and this may help explain the wide variation in the detection of HPV subtypes. Fettig et al. and Bagan et al. detected no

association of PVL with HPV infection [9, 10]. In contrast, Campisi et al. found high-risk HPV in 14 of 58 PVL lesions (24.1%) but did not find any significant difference between PVL and conventional oral leukoplakia for risk of HPV infection [8]. Gopalakrishnan et al. noted HPV expression in 2 of the 8 (25%) p53-positive PVL lesions [14]. In Palefsky et al. study, 8 of the 9 lesions (88.9%) expressed HPV infection [3]. Femiano et al. confirmed the expression of HPV 11, 16 and 18 in 50 PVL lesions [29]. These findings might suggest either no consistent association between PVL and HPV, or discrepancies in the selection of probe sequences and in sensitivity of the detection techniques. Additional factors which may be considered include viral factors like viral load, HPV latency and host immune responses including humoral, cellular and innate immune status [30]. In order to evaluate the discrepancies observed in high-risk HPV and PVL association, it may be worthwhile to investigate the differences in the immune response to HPV infection, and natural polymorphisms or genetic variations between individuals in immune-related genes.

p53 is frequently mutated in HPV-negative squamous cell carcinomas of the vulva, whereas in HPV-positive lesions, it is inactivated by the viral oncoprotein E6 [31]. Lee et al. showed a high rate of malignant transformation in oral leukoplakia patients demonstrating p53 overexpression [32]. Its overexpression in oral cancer was shown to present with poor prognosis and recurrence [33, 34]. We did not evaluate the mechanism associated with the overexpression of p53 gene, i.e., binding of wild-type p53 by an intrinsic protein like Mdm2, or a viral protein like HPV-E6, versus mutation of the p53 gene. Molecular studies involving higher number of PVL cases will be of help in determining the mechanism of p53 overexpression.

Different treatment modalities have been tried for PVL patients. Surgery and laser ablation are the most commonly used methods. The patients in Hansen et al. study were most commonly treated with radiation and surgery, but irrespective of the type of treatment, PVL could not be completely eliminated [1]. Surgery and laser ablation were used in 80 and 60% patients by Bagan et al., but recurrences were detected in 86.7% of patients and new lesions developed in 83.3% cases [22]. Fettig et al. found that both simple excision and laser ablation were ineffective, and ultimately, performed local block resections to prevent recurrences [9]. Other studies reported obtaining similar results in PVL patients [21, 23, 35]. Poveda-Roda et al. noticed an improvement in 38.8% patients by the use of topical or systemic retinoic acid, but the lesions recurred upon discontinuation of the treatment [36]. Many of the treated patients exhibited adverse reactions like desquamation and pruritis. The lesions could not be completely eliminated by surgery and/or laser ablation in any of the patients in our study. Almost all of

them had recurrent or persistent lesions in the same and/or different sites.

Conclusion

In our study, 45% patients with PVL developed malignant lesions. We found no definite association between high-risk HPV and PVL. The patients developed recurrent PVL lesions at the same sites with few patients demonstrating additional sites of involvement. High recurrence rates and relentless progression to malignancy remain the risk factors for PVL. Thus, early detection with multiple biopsies done over several years and long-term close clinical follow-up are essential to facilitate effective management of the condition. Given the recalcitrant, aggressive and malignant potential of PVL, all oral health care providers, including oral pathologists, head and neck pathologists, general practitioners and otolaryngologists should be sentient of this condition. Additional molecular and biochemical studies are required for enhanced understanding of the etiopathogenesis of PVL, which might lead to advent of new, improved diagnostic and therapeutic approaches.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest or other disclosures.

Ethical Approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent The study was conducted after obtaining an approval from the University of Florida Institutional Review Board.

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