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Abstract: Abstract
Proliferative verrucous leukoplakia is a potentially pre-malignant lesion that undergoes malignant transformation in 40 - 70% of cases. It is multifocal and despite progression depicted along histological grading, it remains difficult to predict which lesion will progress. Its clinical homogeneity allows for theorising a single or small number of molecular pathogenic pathways. This study reviewed the evidence in the literature of the molecular aetiology and pathogenesis of PVL in comparison to the conventional OED.
We systematically searched the literature using the MeSH terms ''proliferative'' ''verrucous'' ''leukoplakia''. The Cochrane systematic review protocol was adopted. In all, 19 papers from 43 original articles met the inclusion criteria with 13 proteins assayed in 344 tissues. IHC, PCR and ISH were used for oncoviral DNA and human DNA alteration detection. TP53, p14ARF and p16INK4A (coded by CDKN2A locus) were investigated. All studies defined their research objectives with clearly stated outcomes.
This review showed that PVL transformation did not follow the same pathway as OED. Weak evidence were generated suggesting possible correlations of DNA aneuploidy, LOH at locus 9p21 and specific MCM protein expression, to PVL transformation. Also, other critical pre-malignant to malignant transformation pathway studies like COX-2/PEG2 regulation and promoter methylation analysis were either incomprehensibly investigated or omitted. The review clearly showed sparse and out of date studies of PVL samples. It is therefore necessary to undertake further studies that can access the more comprehensive landscape of somatic genomic alterations found in malignancy, to show important or even distinct pathways of this condition.

*Cover Letter

Mr Eyituoyo Okoturo

Molecular & Clinical Cancer Medicine Dept

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Prof D.A. Mitchell

Editor, BJOMS

Dear David,

RE: Molecular Pathogenesis of Proliferative Verrucous Leukoplakia: A Systematic Review

We would be grateful if you will consider our manuscript for publication in BJOMS.

In our continual effort to uncover the pathogenesis of PVL transformation - since 1990, we firmly believe that any further research on the molecular pathogenesis of PVL should be preceded by a documentation of a robust review of the literature, thus our reason for doing this study.

In addition, we ask for a waiver on the 29 references listed in this ''full length paper''. As this is a review with 19 publications mandatorily considered, only 10 additional references were cited to help validate our viewpoints.

We strongly believe that this publication will add to a depleted literature on PVL.

I look forward to your kind reply.

Yours sincerely (on behalf of the co-authors)

E.Okoturo

Eyituoyo Okoturo

BRITISH JOURNAL OF ORAL & MAXILLOFACIAL SURGERY

Author contribution

Manuscript Title: Molecular Pathogenesis of Proliferative Verrucous Leukoplakia: A Systematic Review

Please provide details in the table below of each author(s) contribution to the submitted manuscript

AUTHORS	Conception and design of study/review/case series	Acquisition of data: laboratory or clinical/literature search	Analysis and interpretation of data collected	Drafting of article and/or critical revision	Final approval and guarantor of manuscript
Eyituoyo M Okoturo	√	√	√	√	√
Janet M Risk	√		√	√	√
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Title Page

Title: Molecular Pathogenesis of Proliferative Verrucous Leukoplakia: A Systematic Review

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Molecular Pathogenesis of Proliferative Verrucous Leukoplakia: A Systematic Review

Introduction

Proliferative verrucous leukoplakia is a rare form of oral leukoplakia first described in 1985 by Hansen et al. (1). It manifests initially as an oral white plaque that can be multifocal with confluent exophytic proliferation. It is capable of transforming to verrucous and invasive oral squamous cell carcinoma (OSCC) in 40 – 70% of cases (1, 2). Additional clinical characteristics include a high tendency for recurrence after excision, and notable absence of conventionally recognised environmental risk factors such as tobacco and alcohol (2). There is gender and age prevalence for females in their 6^{th} and 7^{th} decades notwithstanding that twice the number of oral cancers occur in men. Characteristic histories of numerous biopsy episodes with post-excisional recurrence are a prerequisite for histopathological diagnosis. Prognosis is largely poor due to a combined effect of ineffective therapeutic measures (surgery, radiotherapy) and a high rate of recurrence (1, 3).

Despite *Hansen et al*'s (1)10 step grading system, diagnostic, prognostic and therapeutic uncertainties remain.

As has been described in oral epithelial dysplasia (OED) and other epithelial derived neoplasm (4, 5), the pathogenesis from normal epithelium may be the result of a multistep progressive mechanism involving genetic and epigenetic instability often through gene mutation and chromosomal breaks and losses, in particular affecting proto-oncogene and tumour suppressor genes. Delineating those genes implicated and characterising these molecular events with clinicopathological features may provide new insights into the pathogenesis of epithelial derived neoplasm like PVL (5).

The molecular pathogenesis of PVL - OSCC continuum may differ to the highly heterogeneous patterns seen in the more common de novo OSCC spectrum for the following reasons; the high rate (>40%) of malignant progression with twice as much female preponderance (1, 2), the inability

of histopathological grading to predict malignant which lesion will progress (1) and the clinical homogenous phenotype that typifies PVL. This leads to speculation that there are single or a small number of critical molecular aberrations common to all or most PVL cases.

The aim of this review is to systematically audit the molecular aetiology and progression of PVL as described in the literature and to compare and contrast with the conventional OED.

Material and methods

Our search methodology was modelled after the Cochrane systematic review protocol (6). The MeSH phrase "proliferative verrucous leukoplakia" was used for the initial search. Articles were identified using the search tools; Pubmed (1950-2017), Ovid Medline (1946-2017) and Web of science (1900-2017). Additional articles were identified from the reference list in these papers.

English language article titles and abstracts of papers identified through the above described search process were read to identify papers suitable for the present review. We only considered papers exploring the molecular nature of PVL. Reviews, meeting abstracts and clinical management related articles were excluded. The quality of selected full articles were assessed using a modified 10 step (cross sectional study) or 11 step (case controlled study) National Institute of Health (NIH) quality assessment tool (QAT) questionnaire (6).

The algorithm of QATs used for this review and indeed others, do not qualitatively assess experimental or laboratory methods for protein assay / DNA detection. As this was deemed a necessity for this review, an attempt was made to evaluate their robustness by assessing their experimental design and use of the experimental controls.

Results

The study search protocol identified 88 publications comprising 43 original articles, 11 meeting abstracts, 20 review articles, 6 letters, 5 notes, 2 non-English articles and a repeat publication. A total of 19 of the 43 full papers met the above search criteria (Table 1). All 19 papers were subjected to the NIH QAT. In summary, 13 proteins were assayed in 344 tissues from 242 subjects at a number of samples per study median of 14. Immunohistochemistry (IHC), PCR and in-situ hybridisation (ISH) were used for oncoviral DNA and varying human DNA alteration detection protocols. Assessment for DNA ploidy and screening for fungus was also done. The genes investigated were *TP53,* $p14^{ARF}$ and $p16^{INK4A}$ (coded by *CDKN2A* locus)*.* All studies defined their research objectives and outcomes, as shown in Tables 2 & 3.

Infectious aetiology

Oncogenic Viruses: Ten studies explored the potential role of an oncogenic virus in PVL (7-16). Three groups of viruses were investigated - papillomavirus, polyomavirus and herpesvirus. Seven of the ten studies (8, 9, 11-15) co-amplified *degenerate* primers in PVL. Five of the seven co-amplified sub-type specific primers for viral DNA detection while, the remaining 2 of the 7 studies used ISH (14, 15). The 3 remaining papers (7, 10, 16) considered strong p16 IHC nuclear and/or nuclear with cytoplasmic staining, as a surrogate for high risk (HR) HPV oncogene expression presence in OSCC. In addition, Borgna et al (10) combined p16 IHC assessment with HR HPV DNA ISH, seeking evidence of DNA from any of 12 high risk HPV types. Bagan et al (9) demonstrated the presence of EBV DNA in 60% (6/10) of PVL samples while 3 others demonstrated the presence of HPV 16 DNA in 78% (7/9), 20% (2/10) and 57% (4/7) of PVL samples (14-16). Thennavan et al, (16) reported a negative p16 expression for their singular case of PVL related OSCC. Unsurprisingly, these 4 studies were not able to demonstrate provided influence of an infectious agent on PVL pathogenesis. Perhaps, it is more

significant that the remaining 6 studies detected no virus in their PVL samples.

Fungal detection

Silverman et al. (2) screened for candida in PVL samples using Candida bromo cresol green (BCG) agar medium, periodic acid Schiff (PAS) and speciation germ tube testing. Despite the speciation test revealing the presence of *Candida albicans* in 50% (19/38) of samples with 68% (13/19) of these samples undergoing malignant transformation, the authors reported that this did not reveal any evidence of influence on PVL.

Potential Host Biomarkers

p53 (*TP53*):

Five studies examined p53 expression in PVL using IHC. Four of the five studies used the more sensitive indirect IHC method (streptavidin-biotin and avidin-biotin complex) while the 5th used the less sensitive direct IHC method. Their scoring was based on comparative nuclear positive and nuclear negative staining areas (7, 12, 14, 17, 18). In addition, Gopalakrishnan et al. (14) investigated for potential *p53* mutation by single strand conformational polymorphism (SSCP). All studies showed high expression of p53 in PVL samples when compared to their matched OSCC. However, there was no evidence of *TP53* mutation. The authors concluded that there was no evidence of specific association between p53 expression and PVL or with progression to OSCC.

Genomic alterations

Kersty et al. (18) screened for genetic alteration in the CDKNA2 genes $p16^{INK4a}$ / $p14^{ARF}$ in formalin fixed paraffin embedded (FFPE) PVL samples using mutation analysis, allelic imbalance and homozygous deletion methodologies.

Allelic imbalance: Three fluorescence labelled microsatellite markers on 9p21 were co-amplified in PVL and matched normal tissues. Scoring was done by comparing ratio of PVL and control DNA allelic pair using an

automated sequencer and scanner. They reported that allelic imbalance occurred in 45% (9/20) of PVL samples.

Mutation analyses: INK4a/ARF specific exons, 1α, 1β and the common exon 2 of the *CDKNA2* gene were amplified using intron based primers. SSCP followed by sequencing revealed exon 2 SSCP base changes in 20% (4/20) of PVL samples which when sequenced gave 5 base changes. All 5 were in the coding region of p16 and 3 in the coding region of p14.

Homozygous deletion: HPRT gene was co-amplified with the exon of interest and the PCR product was electrophoresed for deletion assessment. The authors reported homozygous deletion of exon 1β of the p14 gene in 40% (8/20) of PVL samples and 35% of exon 1α (7/20). A total of 25% (5/20) of the PVL samples had both exons 1α and 1β deletion while exon 2 had no deletion.

The study showed for the first time that there are frequent (16/20 samples) gene alteration at the INK4/ARF locus in early PVL lesions, although 4 of these were detected by LOH alone and may not be targeted to the CDKNA2 gene (18).

DNA ploidy: Kahn et al, Klanrit et al and Gouvea et al studies (19-21) investigated DNA ploidy in FFPE PVL-OSCC continuum samples utilising different flow cytometry protocols. Samples were micro sectioned, nuclei extracted, fluorescently labelled and assessed for DNA content by flow cytometry. The DNA ploidy status was assessed by percentages of diploid and aneuploidy cell and nuclei population within the cell cycle. Their results were quantified as a histogram in which the fluorescence emitted was directly proportional to individual DNA content. Kahn et al. and Klanrit et al. reported abnormal DNA ploidy in 100% (4/4 & 6/6) with 50% (2/4 & 3/6) of cases showing diploid cells in the early grade PVL samples respectively. Gouvea et al. reported 95% (20/21) abnormal DNA ploidy with 4.8% (1/21) of cases showing diploid cells in early grade PVL samples. This author's non-matching control samples showed a 100% (12/12) diploid status.

All 3 papers suggested by samples, an association between increased DNA aneuploidy and increase degree of epithelial dysplasia.

Other protein expression Studies

MCM2, MCM5: The MCM (mini chromosome maintenance) complex regulates the process of DNA replication "licensing" at origin of replication. Two studies examined the expression of MCM2 and MCM5 in PVL using indirect IHC (17, 21). Their scoring was based on the ratio of number of positively stained cells to total number of epithelial cells. All cases were found to be positive for MCM (12/12 and 21/21) with grade of dysplasia correlating with strength of protein expression (3/12) and (12/21; p=0.03). Both studies suggested that higher MCM positivity in mild to moderate dysplastic PVL could predict malignant transformation.

Other protein targets: We identified 8 PVL studies that investigated the expressions of Ki67 (7, 12, 16, 17, 21), α-*SMA* (22)*,* geminin (21),TGF- α (23)*,* interleukin-6 (IL-6) (24), BCL2, COX-2 & CD34 (16). Bagan et al. (24) assessed IL-6 in blood and saliva samples using indirect enzyme linked immunosorbent assay (ELISA) while the others used tissue samples and IHC for assessment. All IHC scoring was based on the ratio of the number of positively stained nuclear or cytoplasmic cells to the total number of epithelial cells, with the exception of Thennavan (16) who considered positively stained microvessels in an endothelial cell population as positive for CD34. All IHC assayed proteins revealed a mild to moderate levels of expression that did not correlate with increasing levels of dysplasia. The saliva and blood levels of IL-6 was reportedly lower in PVL than in the OSCC group but higher than the control group of non-matching normal mucosa (p=0.01) (24).

Discussion

The various studies have investigated PVL pathogenesis and protein expression, viral antigen detection / DNA alterations and sought to

determine any correlation. Overall, it was impossible to show that PVL transformation followed the same pathway as OED (5).
While some of the methodologies used may have been contemporary for the period in which they were undertaken, newer techniques are required to investigate further. In the papers reviewed, 9 of the 17 controlled-studies employed both positive and negative controls for their assay/protocol to ensure sensitivity and specificity. It was difficult to establish standardisation of protocol and quality of the outcomes of the 2 studies with no laboratory controls (2, 20).
In terms of the strength of the methodology and their supporting evidence, eight of the nine IHC studies used the indirect IHC method that is considered as more sensitive, as the labelled secondary antibody employed allows for amplification by multiple binding sites to the antigen-antibody complex (25). Furthermore, PVL studies using comprehensive proteomic approach like mass spectrometry based methods, may have benefits over single protein assay.

Due to the multifocal yet relatively homogenous characteristics of PVL, infectious aetiology has been suggested (15). On balance, the evidence does not identify a role for oncogenic virus in PVL. qRT-PCR of E6/E7 mRNA is regarded as the gold standard for HPV detection in HNSCC as it provides a better indication of the role of HPV as a potential agent than simple DNA detection (26). None of these studies used this gold standard. Similarly, 3-5% of OSCC have transcriptionally active HR HPV subtypes (27). In this regard, the conclusion of correlation between HPV and PVL pathogenesis reached by the authors can now be re-appraised.
Genomic instability is a known pathogenic route that characterises the early steps of malignant transformation by creating increased disposition to gene mutation (4). p53 gene is reported to be the most frequently altered gene in HNSCC (28). While p53 gene mutation and protein overexpression are frequently closely related events, the data of PVL reviewed here did not detect any p53 gene mutation. The IHC methods

used could not distinguish between wild and mutant type *TP53* indicating that the positive staining could be attributable to wild type or mutant protein expression (12, 14). Furthermore, the *TP53* mutation screen method used is known to be of limited utility and sensitivity and has been completely superseded by more modern technologies.

Studies of another common potentially carcinogenic process focused on the INK4a locus and revealed frequent LOH (45%) of the 9p21 region (18). Clearly, future studies using more sensitive, genome wide – based methods in array comparative genomic hybridisation (aCGH), SNP arrays or next generation sequencing (NGS) can investigate this further.

Chromosomal instability detected as aneuploidy i.e. numerical / multiple structural chromosomal anomalies, is the commonest form of genomic instability in cancer (4, 18). Thus, it comes as no surprise that amongst the early research of PVL pathogenesis, several attempts were made to assess aneuploidy using cytometry (19-21). However, while correlated DNA flow cytometry based on DNA content, gives a reasonable overview of DNA stability, it offers little data at the gene level (19-21). Clearly, genome wide base studies can investigate this further.

Another reported critical pathway of pre-malignant to malignant progression is the overexpression of COX-2, a synthases for prostaglandin E2 (PGE2) (4). COX-2/PGE2 regulates proliferation, migration, invasion, angiogenesis and induction of vascular endothelial growth factors (VEGF). Surprisingly, only one PVL paper has to date examined COX-2/PGE2 regulation (16).

In addition to the studies described above, there remains some major omissions in the analysis of PVL. For example, to date there have been no published studies of epigenetic analyses. Like its genomic counterpart, epigenetic dysregulation can be carcinogenic and indeed its role in OED transformation has been established (29).

Detection of this instability across a PVL continuum could allow for early or a late event categorisation, an aspect currently being researched at our institution.

In conclusion, now that we have ready access to the more comprehensive landscape of somatic genomic alterations found in malignancy, it is clear that there have been only very limited, and often now, outdated studies of PVL samples with no clear picture. Weak evidence has been generated suggesting possible roles for chromosomal alterations leading to aneuploidy, LOH at locus 9p21 and differences in specific MCM protein expression but these clearly require further investigation.
The generation of such comprehensive data might have wider implications beyond better understanding of PVL, illuminating important or even distinct pathways for this rare condition, but also for HNSCC and other tumour sites.

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Table 1. Relevant publications

	Publications	Study Design	Biopsy Sample/ Center	Protein/DNA aberrations	Correlation to PVL
1.	Kahn et al 1994[19]	Retrospective cross sectional	27/single center	DNA ploidy	+ve DNA ploidy link
2.	Palefsky et al 1995[15]	Retrospective case controlled	9/single center	Virology HPV DNA	+ve HPV
3.	Kannan et al 1996[23]	Retrospective case controlled	10*/single center	TGF- α	+ve TGF- α
4.	Gopalakrishnan et al 1997[14]	Retrospective Case controlled	10*/single center	*TP53*, p53, HPV DNA	-ve *TP53* mutation +ve p53 & HPV
5.	Silvermann & Gorsky 1997[2]	Longitudinal Study	38*/single center	Candida albicans	+ve C. albican
6.	Fettig et al 2000[12]	Retrospective Case Controlled	10*/single center	HPV DNA, p53, Ki67	+ve p53 & Ki67 -ve HPV
7.	Campisi et al 2004[11]	Prospective Case controlled	58*/dual-centers	Virology HPV DNA	-ve HPV
8.	Bagan et al 2007[8]	Prospective Cross sectional	10*[@]/single center	Virology HPV DNA	-ve HPV
9.	Klanrit et al 2007[20]	Retrospective Case controlled	6*/single center	DNA ploidy	+ve DNA ploidy link
10.	Bagan et al 2008[9]	Prospective case controlled	10*[@]/single center	Virology EBV DNA	+ve EBV

11.	Kresty et al 2008[18]	Retrospective Case controlled	20*/single center (LOH, homzygous deletion)	*CDKN2A*, p53	+ve p^{14}, p^{16} link
12.	Gouvea et al 2010[17]	Retrospective Case controlled	18[@]/single center	p53, Ki67, MCM2 MCM5	+ve MCM2/5 link
13.	Gouvea et al 2013[21]	Retrospective Case controlled	65/dual-centers	DNA ploidy, MCM2 Ki67, geminin	+ve DNA ploidy & MCM2 link
14.	Garcia-Lopez et al 2014[13]	Retrospective case controlled	10*/single center	Herpes, papilloma & polymaviruses	-ve viral DNA
15.	[@]Thennavan et al 2015[16]	Retrospective case controlled	7 / single center	Ki-67, p16, CD34 Bcl-2, COX-2	+ve Ki67, p16, COX-2, CD34
16.	Akrish et al 2015[7]	Longitudinal Study	38/single center	p53, p16, Ki67 HPV DNA	+ve p53
17.	Bagan et al 2016[24]	Prospective case controlled	20*/single center	IL-6	+ve IL-6 link^
18.	Akrish et al 2017[22]	Longitudinal study	32[@]/single center	α -SMA	-ve α-SMA
19.	Borgna et al 2017[10]	Cross sectional Study	15 /single center	p16, HPV	-ve HPV

*: Sample sizes same as number of patient

^: Serum and saliva markers, [@]: Overlapping cohort / sample set

Table 2. NIH QAT Scores

	Publications	Assays/Protocols	Laboratory Controls +ve	Laboratory Controls -ve	QAT Scores
1.	Kahn et al 1994	(Flow cytometry)	✓	-	3/10
2.	Palefsky et al 1995	(PCR, ISH)	✓	-	8/11
3.	Kannan et al 1996	(IHC*)	✓	✓	6/11
4.	Gopalakrishnan 1997	(IHC*, SCCP, PCR, ISH)	-	✓	4/11
5.	Silvermann & Gorsky	BCG agar media	-	-	5/10
6.	Fettig et al 2000	(PCR, ISH, IHC)	✓	✓	6/11
7.	Campisis et al 2004	(PCR)	✓	✓	6/11
8.	Bagan et al 2007	(PCR)	✓	✓	3/10
9.	Klanrit et al 2007	(Image cytometry)	-	-	6/11
10.	Bagan et al 2008	(PCR)	✓	✓	5/11
11.	Kresty et al 2008	(IHC*, PCR-SSCP, DNA Seq)	✓	-	6/11
12.	Gouvea et al 2010	(IHC*)	-	✓	7/11
13.	Gouvea et al 2013	(IHC*, Image cytometry)	✓	✓	7/11
14.	Garcia-Lopez et al 2014	(PCR, ISH, Seq)	✓	✓	7/11
15.	Thennavan et al	(IHC*)	✓	-	5/11
16.	Akrish et al 2015	(IHC*)	✓	-	5/10
17.	Bagan et al 2016	(ELISA)	✓	✓	6/11
18.	Akrish et al 2017	(IHC*)	✓	-	5/10
19.	Borgna et al 2017	(IHC*, ISH)	✓	✓	5/10

PCR-Polymerase chain reaction, **IHC*-** Indirect Immunohistochemistry**, SSCP-**Single strand conformational polymorphism,**ELISA-**Enzyme link immune-sorbent assay, **DNA Seq-**DNA Sequencing, **ISH**-Insitu-hybridisation

Table 3. Modified NIH-QAT questionnaire - Publications in Serial Order

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1. Was the research question or objective in this paper clearly stated and appropriate?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2. Was study population clearly specified & defined?	0	1	1	0	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1
3. Did the authors include a sample size justification?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4. Were all subjects selected or recruited from the same or similar population (including the same timeframe)?	0	1	1	0	1	1	1	0	1	0	1	1	1	1	0	1	0	1	1
5. Were the definitions, inclusion/exclusion criteria, valid & implemented consistently across all the study participants?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6. Were the cases clearly defined and differentiated from controls?	NA	1	1	1	NA	1	1	NA	1	1	1	1	1	1	0	NA	1	NA	NA
7. Were the cases and/or controls randomly selected from those eligible?	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
8. Were the investigators able to confirm if the interest occurred prior to the development of the condition?	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
9. Were the assessors of interest blinded to the case or control status of participants?	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
10. Were key potential confounding variables measured and adjusted statistically in the analysis?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11. Were the interest(s) measured prior to outcome(s) being measured?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Total	**3**	**8**	**6**	**4**	**5**	**6**	**6**	**3**	**6**	**5**	**6**	**7**	**7**	**7**	**4**	**5**	**6**	**5**	**5**

NA: Not applicable