

**Full Title: A Systematic Review of the Genomics of Precancerous Oral Verrucous Lesions**

**Short title:** Genomics of oral verrucous lesions

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## Highlights

- Copy number alterations are shared between OVH and OVC.
- 45% of PVLs showed a mutational event involving both p16 and p14 transcripts of *CDKN2A*.
- In POVLs, high LOH in multiple arms was noted at presentation itself.
- Transition of OVH to OVC involved increases in LOH at 9p ( $p=0.062$ ) and 4q ( $p=0.053$ ).
- Transition from OVH to OSCC involved increases in LOH at 4q ( $p=0.041$ ) and 17p ( $p=0.037$ ).

## Abstract

Precancerous oral verrucous lesion (POVL) is a rare oral precancer entity characterised by multifocal origin and unpredictable long-term evolution to oral squamous cell carcinoma (OSCC) or oral verrucous carcinoma (OVC). There are currently no predictive biomarkers in clinical use. We aim to explore the genomic profile of POVL. 685 POVLs in 26 studies were included in this systematic review - 505 oral verrucous hyperplasias (OVH), 153 proliferative verrucous leukoplakias (PVL), and 27 atypical verrucous hyperplasias (AVH). Genomic data were presented in 15% of studies and biomarker analysis in 85% of studies.

At first presentation, POVLs are characterised by high Loss of Heterozygosity (LOH) (similar to OSCC) and low CNAs. Early acquisition of LOH at multiple arms differentiates OVH from the progressive LOH observed in oral epithelial dysplasia (OED). In its second stage, more CNAs and mutations in *CDKN2A* and alterations to *ELAVL1* expression are noted, but no *TP53* mutations are identified.

The transition from OVH to OVC involves an increase in LOH at 9p and 4q, and from OVH to OSCC increases in LOH at 4q and 17p. Lower LOH at 17p is identified in OVH compared to OSCC ( $p=0.037$ ). Deletions in chromosomal loci 17q12, 5q31.1 and amplifications in 7q11.2, 7q22 are shared between OVH and OVC. PVL shows CNAs at 11q31 and *CTTN* gene may be important in transition. WNT signalling pathway genes *FOLR3* and *SUZ12* are enriched in CN altered regions of POVLs. POVL stroma shows significantly lower  $\alpha$ -SMA and higher CD34 expression compared to OVC and OSCC.

## Background

Head and neck cancer is the eighth most common cancer in the UK (2014), accounting for 3% of all new cases [1]. The majority of head and neck cancer patients (62%) are diagnosed at a late stage [1]. Oral squamous cell carcinoma (OSCC) evolves conventionally from oral epithelial dysplasia (OED), which is a precancer with histologically recognisable atypical cytological changes. In contrast, premalignant oral verrucous lesions (POVL) are slow-growing, multifocal, progressive, hyperkeratotic lesions with a characteristic exophytic growth profile demonstrating architectural atypia rather than conventional cytological atypia seen in OED [2]. Although OED and POVL are both premalignant lesions, the two entities behave differently. Whilst OEDs show a somewhat histologically predictable pathway of malignant transformation depending on exposure to carcinogens and the grade of dysplasia, POVLs display an abrupt and histologically unpredictable evolution; some POVL lesions have an indolent course and may never transform to carcinoma. POVL-associated OSCC (p-OSCC) shows a mortality rate of 30-40%, requiring radical surgery with a high rate of recurrence (>80%) [3] [4 5]. Early recognition will be the key to successful management. OEDs are a heterogeneous mixture of entities, and it is important to separately study distinct entities such as POVLs.

POVLs broadly encompass the disputed terms oral verrucous hyperplasia (OVH), atypical verrucous hyperplasia (AVH) and proliferative verrucous leukoplakia (PVL). Shear et al. first described OVH using the term 'verrucous hyperplasia,' highlighting the benign histological appearance of the first presentation of POVLs [6]. In 1985, Hansen first introduced the term PVL as a "continuum of hyperkeratosis ranging from a simple hyperkeratosis at one end to invasive squamous cell carcinoma at the other" [7]. World Health Organisation (WHO) recognises PVL as a rare, oral, potentially malignant disorder that occurs in older (>60) patients with a female to male ratio of 4:1, some of which transform into oral verrucous

carcinoma (OVC) or OSCC [3]. When OVH presents at multiple sites, the lesions start to show more atypical architecture, which progressively becomes more detectable histologically, and some clinicians prefer to use the term AVH to express the more predictable malignant potential of these recurrent lesions. PVL is a clinical term used retrospectively to denote these recurrent atypical verrucous lesions. At present, there are no defined WHO criteria to diagnose POVLs [3]. Due to their overlapping histological features and the lack of consensus in diagnostic criteria fuelled by the confusing terminology used across the globe, POVLs have been a diagnostic enigma and a management dilemma [8]. OVC is categorised by WHO as a less aggressive subtype of OSCC with an endophytic component in addition to the exophytic profile seen in OVH [3]. OVC is a close histologic mimic of POVLs, and these will be compared in this review.

## **Objectives**

The current knowledge of POVL genetics is in its infancy. We aim to systematically review all the articles published on POVL genomics to collate the current evidence for an identifiable genomic profile of the high-risk POVLs which transform into malignancy. We endeavour to identify affected genes, pathways, and associated copy number alterations (CNAs) and loss of heterozygosity (LOH) events. These data have potential utility for biomarker development, early identification, and successful management of a currently underdiagnosed and mismanaged oral precancer type.

## Methods

### Study Design

The review was conducted according to the centre for reviews and dissemination (CRD) guidance on systematic reviews [9]. The report adheres to the preferred reporting items for systematic reviews- PRISMA [10 11] [12] [13]. The study was registered on PROSPERO [14].

### Search strategy

The major electronic databases, including MEDLINE (Ovid), EMBASE, SCOPUS, Web of Knowledge, and CINAHL, were searched for relevant published literature using text terms oral verrucous hyperplasia, verrucous hyperplasia, proliferative verrucous leukoplakia, proliferative verrucous hyperplasia, verrucous hyperkeratosis, verrucous dysplasia, atypical verrucous hyperplasia, and oral verrucous carcinoma. A total of 1667 citations were identified. And after duplicates were removed, the total citations from all the databases were narrowed down to 397. Due to the rarity of OPVLs, the titles and abstracts of all the 397 articles were reviewed by the main reviewer to select 77 articles for further review (**Figure 1 - PRISMA flow diagram**). The study search was completed in 2018.

### Inclusion and exclusion criteria

Please refer to the inclusion and exclusion criteria displayed in **Table 1**. Due to the complexity associated with POVLs on their own, it was decided that OVC should be excluded from the study. The precancer variants would be compared with transformed OSCC and OVC lesions instead. Restricting the inclusion criteria for specified genetic studies narrowed down the studies to four and was not useful for obtaining a broader picture of disturbed molecular pathways. Therefore, it was decided to include studies discussing molecular markers using well-established lab techniques such as immunohistochemistry (IHC). Due to the current proven evidence of DNA ploidy imbalance in OVH and PVL, studies on DNA ploidy analysis

were excluded from the analysis [15]. In addition, since several studies have discussed and disproved HPV viral aetiology, these were also excluded [16 17].

From the 77 articles, 45 were excluded: 21 were only on verrucous carcinoma without reference to verrucous hyperplasia, 9 had no genetic or gene expression information and either discussed malignant transformation rate or clinicopathological reviews, 3 discussed DNA ploidy analysis, 8 were on oral SCC only, 4 were on HPV aetiology. Of the remaining 32 articles, 6 articles were excluded with the reason (2-conference abstracts with insufficient information, 1-HPV aetiology, 1-OVC NGS analysis, 1-article in a foreign language, 1-malignant transformation rate only), leaving 26 studies for inclusion.

There were two other published systematic reviews on PVL at the time of our assessment [18] [19]. Both these were based only on PVL molecular markers and were excluded from the review, but comparisons are made in the discussion.

### **Quality assessment strategy**

The methodological quality of the studies included in the review was assessed according to the National Institute of Health (NIH) Quality Assessment Tool (QAT) for Observational Cohort and Cross-Sectional Studies questionnaire [20]. All studies with a QAT score above 5 were considered for the review (n=26/26).

### **Data processing and analysis**

From the 26 studies included in this review, data were extracted by one reviewer using a standardised data extraction form and recorded electronically using Microsoft Excel®. This was checked independently for accuracy by a second reviewer. All the bibliographic references were stored using EndNoteWeb online software (<https://access.clarivate.com/login?app=endnote>) database [21].

## Results

### Study characteristics

A total of 685 cases from 26 studies were included in this review (**Tables 2 & 3**). There were twelve articles on 153 PVL cases [22-33] and twelve articles on 505 OVH cases [34-45], and two articles on 27 AVH cases [46-47]. The mean age for all POVLs was 63.9 years (range: 14-95 years; (**See Table 3**) in keeping with >60 age for the presentation of these lesions [3]. The male to female ratio was 1.3:1. Three studies had male-only samples [39-42-45] and one study had female-only samples [26].

On the assumption of possible differences in the genetic composition, the POVLs were analysed separately as two entities to assist in comparisons of the evolutionary pathway: (1) OVH (505 cases of early presentation of POVL) and (2) PVL/AVH (180 cases of late and more progressive presentation of POVL). The majority of the studies were cohort, case-control, or cross-sectional studies, and most compared POVLs with OVC and OSCC, while some compared with normal oral mucosa (NOM) and OED. Some authors discussed transformed OSCC from PVL (p-OSCC) [33-48]. Four studies (n=4/26; 15%) generated genomic data such as CNAs using SNP arrays, targeted gene sequencing, whole-exome sequencing (WES), and microsatellite analysis [22-34-35-47]. There were no studies analysing whole-genome sequencing data. Two of these studies assessed somatic mutations and allelic imbalances [22-35], whilst two studies assessed CNAs using WES [34-47]. The remaining studies (n=22/26; 85%) assessed biomarkers mostly using the percentage labelling index (LI) of IHC to predict gene expression levels (**Table 2**). Whilst these biomarkers could be important in tracking clonal evolution, the expression levels would not always be indicative of gene alterations.

## Data from genomic studies

### 1. Copy number alterations

There were two studies which discussed CNAs, Samman et al., 2015 [34] and Wu et al., 2018, [47]. In Samman's study, frequency karyograms of the CN analysed sample were produced using an in-house built programme that utilised BED files (.bed). The threshold was 0.05 (above-gain, below-loss) [34]. According to **Figure 2** based on Samman's study, OVH and OVC share CNAs, but more CNAs are present in OVC compared to OVH [34]. OVCs showed low levels of deletions, which suggests that upregulation of oncogenes may be important in OVC development [34]. OVH CN gains mapped at 7q11.2 (50%), 7q22 (50%) and OVC CN gains mapped at 7p22, 7q11.2 (50%), 7q22 (50%), 15q15 (30%), 3p21 (30%), 16q22 (25%) 17q23 (25%). OVC CN losses-17q12- 50%; 6p21- 25% [34].

In addition, the GISTIC (Genomic Identification of Significant Targets in Cancer) algorithm was used in Samman's study to identify significantly altered somatic driver CN alterations using the amplitude and the frequency [49]. In OVH patients, two deletion points 5q31.1 (20%) and 17q12 (20%) were significantly altered on the GISTIC plot, but no significantly altered amplification regions were detected [34]. In OVC patients, 5q31.1 (15%), 6p21.2. (21%) and 17q12 (15%) were identified as the significantly deleted regions whilst six significantly altered amplification regions were identified- 3p21 (50%), 7p22 (75%), 7q11.2 (70%), 7q22.1 (35%), 15q15 (40%), 16q22 (40%), 17q23 (45%) [34]. The frequency difference in visual examination of the karyograms and the GISTIC analysis findings was attributed to the human eye errors with no exact frequency percentages for analysis.

When the above data from GISTIC CN plots and the CN karyograms were further compared, deletions in chromosomal loci 17q12, 5q31.1 and amplifications in 7q11.2, 7q22 appear to be shared between OVH and OVC. Therefore, these CNAs are considered to play a pivotal role in the transformation of OVH to OVC.

Wu et al. identified 125 CNAs in two OVH cases which transformed to OSCC [47]. Although this was the largest prospective cohort study to date with 269 OVH cases, only 2 cases of progressive OVH (considered to be PVL) were analysed for CNAs. Common amplifications identified were at 3p21, 6p21, 9q33, 10q26, 11q13, 13q34, 14q22, 15q21, 16p13, 17p13, 18q12, 19p13, 19p12, and 22q12 loci [47]. 3p21 amplification is shared between OVCs in Samman's study and progressive OVHs in Wu's study, suggesting that this may be an important event in the evolution of OVH to OVC. However, Wu et al. did not identify any of the CNAs seen in OVH samples of Samman et al. study.

## 2. Loss of heterozygosity (LOH)

Two studies that reported allelic imbalances in OVH and PVL are displayed in **Figure 3** [22] [35]. One study on 20 PVL cases reported high LOH making this the most frequent molecular alteration detected in PVL [22]. In the other study on 25 OVH cases, LOH was noted at an earlier stage; whilst the LOH profile of OVH was similar to high-grade OED and OSCC, the early acquisition of allelic losses at multiple arms differentiated them from LOH observed in OED to OSCC [35]. There was a significantly higher incidence of LOH in OVH compared to NOM (60%), but lower levels were noted compared to OVC (100%) and OSCC (95%). This was notably seen at the 9p21 locus, which is associated with oral cancer progression and recurrence [50 51]. The transition from OVH to OVC involved increases in the incidence of LOH at 9p ( $p=0.062$ ) and 4q ( $p=0.053$ ) [22]. The transition from OVH to OSCC involved statistically significant increases in LOH at 4q ( $p=0.041$ ) and 17p ( $p=0.037$ ) [35].

## 3. Candidate genes, mutations, and gene pathways

Mutations in *CDKN2A* (the gene encoding p16INK4A and p14ARF) were detected in 20% of PVL cases, indicating possible utility in predicting malignancy [22]. p16INK4A and p14ARF will be referred to as p16 and p14 in the text. The study concluded that mutational events were less common than LOH or deletion events in PVL. In PVL, p14 exon 1 $\beta$  was deleted in 40%

of cases; 1 $\alpha$  was deleted in 35% of cases [22]. 60% of cases with 1 $\beta$  deletions also had 1 $\alpha$  deletions but without homozygous deletion of shared exon 2 [22]. Some of these changes reached levels reported in advanced OSCC [52, 53]. It appeared that p14 plays a major role in PVL lesions compared to other OED. Moreover, Kresty et al. reported different p16/p14 inactivation events in PVL compared to non-PVL oral dysplasia [54]. 45% of PVL cases showed a deletion or mutational event involving both p16 and p14 transcripts of the *CDKN2A* gene, whereas non-PVL dysplasia only demonstrated a 15% incidence of concomitant alterations affecting both transcripts [22 54]. In PVL, the area of highest mutations was exon 2 [22].

No *TP53* gene mutations were identified in any study, although p53 immunohistochemical overexpression was noted in 78% of POVLs [23]. Some concluded that this could be related to an imbalance in cell cycle proteins such as p14 and p16 since it is involved in the stabilisation of p53 by inhibiting MDM2 [22]. The work by Kresty and colleagues suggested that there are deletions involving the p14 transcript, which may explain this p14 protein imbalance [22]. There was significantly lower LOH at 17p in OVH compared to OSCC. Therefore, it was suggested that this could be because the p53 protein is inactivated by a ubiquitous pathway other than by *TP53* mutation, such as HPV infection [23]. However, currently, there is little evidence to support a viral aetiology for POVL [55].

According to Samman et al., two amplification loci are shared between OVH and OVC -7q11.2, 7q22 [34]. Therefore, these probably play an important role in the transformation of OVH to OVC. *MCM7* gene, at the 7q22 locus, is highly overexpressed in OED and OSCC [56]. Another gene at this locus, *SERPINE1*, has been implemented as a regulator of oral carcinogenesis and might also play a role in OVC development [57 58]. Amplification at the 7q11.23 region has been observed in other carcinomas such as cholangiocarcinoma [59] and papillary thyroid carcinoma in patients with radiation exposure [60]. Some studies have identified the *HIP1*

gene at this site to be overexpressed in colon and prostate cancers [61]. No specific association with head and neck cancer is identified.

16q22.1 region showed recurrent CN gain in OVC (25%) and, therefore, the *CDH1* gene (encoding Epithelial-Cadherin) located in this region could be overexpressed in OVC [34]. Since E-cadherin loss causes discohesion of cancer cells and infiltration in OSCCs, the gain at 16q22.1 in OVC may be the reason why OVCs show a broad and pushing invasive front with limited metastasis [34].

Wu et al. concluded that the expression of eleven head and neck cancer-associated genes significantly correlated with the CN altered loci in progressive OVH -*APOE*, *GAL*, *TOMM40*, *FOLR3*, *TPCN2*, *ORAOV1*, *MRPL21*, *FADD*, *GEMIN7*, *RNF121*, *PPFIA1*[47]. He also concluded that the overexpression of *CTTN*, *FOL3*, *ORAOV1*, *PPFIA1*, and *RNF121* correlated with a reduced overall survival by Kaplan-Meier analysis. This study concluded that amplification of folate receptor gene *FOLR3* is associated with OVH progression [47]. However, a recent study discusses decreased folate receptor expression in OSCC [62]. Further studies are necessary to establish the role folate receptors play in oral carcinogenesis. In addition, Wu et al. reported recurrent amplification of 11q13 (35). CN altered genes, *CTTN* and *ORAV1*, in progressive OVH mapping to the 11q13 region were also observed in OSCC, suggesting that these genes may be important in transformation [63].

Suppressor of zeste 12 protein homolog (*SUZ12* is a cancer gene encoding a polycomb-repressive complex (PCR2 protein) which is important in DNA methylation and epigenetics [64 65]. This gene was identified as a cancer gene shared between OVH and OVC [34]. *SUZ12* is a known cancer-associated gene, the overexpression of which correlates with unfavourable prognosis of colon and oral carcinomas [66 67]. Interestingly, however, in the Samman study, *SUZ12* was identified in the 17q11.2 CN loss region of OVH [34]. *SUZ12* mutation has been implicated in the development of myeloproliferative disease [64], but there were no reports in the literature about the effect of *SUZ12* downregulation in oral carcinogenesis. Interestingly, a

recent study describes how *SUZ12* loss could initiate *NF1* mutations via overexpression of Ras signalling [68]. As a result, *SUZ12* has been identified as a possible therapeutic and prognostic biomarker for *NF1*-associated neurofibromas [68].

In Samman's study, the data from the GISTIC plots were analysed to identify likely somatic driver genes in the most significant amplification and deletion peaks [34]. The resulting genes were further analysed by running against 13 enriched Kyoto encyclopaedias of gene and genomes (KEGG) pathways most related to head and neck cancer, gene census, and Stransky mutation list, which includes 76 previously identified genes in head and neck cancers with statistically significant mutations [69]. The resulting gene hit list included four genes involved in the KEGG WNT signalling pathway (*PP2CA*, *SKP1*, *TCF7*, *WNT8A*), two genes involved in the KEGG cell cycle pathway (*SKP1*, *CDC23*), and one cancer gene (*SUZ12*). Other genes identified are *ILP9* (KEGG JAK-STAT signalling pathway), *WNT8A* (KEGG hedgehog), and *VDAC1* (KEGG calcium signalling pathway). Interestingly, OVH shared all these genes with OVC [34]. It is noteworthy that WNT signalling pathways are identified as an important driver in OSCC by the recent NGS network [63].

## **Data from non-genomic studies**

### **1. Molecular markers / biomarkers**

The main molecular markers assessed by more than one study were p53, p16INK4A (p16), Ki-67, TGF- $\alpha$ , and MCM2 expression. Positive p53 immunohistochemistry labelling was identified in 78% (n=149/191) of the PVL and OVH cases. One major limitation of comparing these studies was the different labelling indices used, which compromised any possibility of collective meta-analysis for this review. One study showed significantly high p53 expression in OVC compared to OVH (p<0.001) [36]. Wu et al. showed that there was a significant correlation between the level of p53 expression and the differentiation status of these lesions [43].

Several studies showed similar levels of Ki67 staining in PVL and OSCC [26 28 31 46]. OVC demonstrated diffuse nuclear staining of p53 and Ki67 compared to OVH [36 46]. A recent study indicates a concurrent increase in p53 and Ki67 could significantly predict the cases of oral leucoplakia, which transformed to OSCC, which may be useful in future studies [70]. Three studies investigated MCM2 expression in POVLs [26 27 38], and two demonstrated significant positive correlations with increasing severity of dysplasia to OSCC [27 38]. Three studies investigating p16 immunohistochemistry expression concluded with negative results [25 31 54]; 50-65% of p16 immunoreactivity was observed in three OVH cases with negative high-risk HPV in situ hybridization [25].

Human antigen R (HuR/ ELAV-like protein 1) is an mRNA-binding protein that is mainly located in the nucleus but travels between the nucleus and the cytoplasm [71]. The ELAVL family of proteins plays a role in stabilizing ARE-containing mRNAs and can regulate gene expression [71]. An increase in cytoplasmic HuR has been implicated in carcinogenesis [72-74]. In head and neck carcinomas, it is considered a mediator of growth and invasiveness of cancer and also an adverse prognosticator [74]. Habiba et al. studied HuR expression in 6 OVH and 25 AVH cases [46]. There was significantly different HuR expression in AVH cases (64%) compared to OVH (1.5%). Interestingly, 6 AVH cases transformed to malignancy, 3 to OSCC, and 3 to OVC, and all cases demonstrated high cytoplasmic HuR expression; none of the cases with low HuR levels transformed to OSCC [46] (additional follow up information through personal communication). This study concluded with high confidence that a >60% labelling index of cytoplasmic HuR levels could predict OVH cases at higher risk of malignant transformation [46]. HuR is encoded by gene *ELAVL1* at chromosome 19p13.2 locus. There is evidence of genomic instability at this locus, where CN amplifications at 19p13 were identified in the Wu et al. study [47]. This finding is consistent with a possible role for HuR in regulating the proliferation of malignant cells [72]. However, no allelic loss was noted at this locus.

Significantly high BUBR1 protein expression in OVH compared to OED was noted in one study with similar expression levels in AVH and OVC [45]. BUBR1 is a spindle assembly checkpoint protein that has been noted as a significant player in oral carcinogenesis [75]. A recent study has shown significantly high BUBR1 protein levels in dysplasia compared to normal mucosa [76]. The *BUB1B* gene, responsible for BUBR1 protein, is located in the 15q15 region, which also showed CN alterations in OVC [34] but not in OVH or PVL [47], suggesting a potential role in the transformation of OVH/PVL to OVC.

The following studies did not have sufficient evidence to explore their significance in POVL progression. One study with a significant difference in TGF- $\alpha$  expression between PVL and NOM [24], one study with non-significant EGFR overexpression in OVH [43], one study on c-erbB-3 protein in OVC [41] type IV collagen and laminin in OVH [44], and a preliminary study on EBV in PVL [29].

## **2. Clonality of POVLs**

The data here is from one conference abstract assessing clonality of PVL lesions and hence should be considered as preliminary evidence [30]. BAC arrays for nine patients with multiple PVL tumours were compared, and the breakpoints of genetic alterations were determined by counting the number of shared breakpoint positions between lesions. Five patients showed a large number of shared genetic aberrations between their PVLs and p-OSCCs [30]. This supports the theory that these multifocal OVH lesions are genetically related and also that p-OSCC might represent a genetically different OSCC subtype.

## **3. Tumour microenvironment of PVL and PVL associated OVC and OSCC**

There is accumulating evidence that the tumour microenvironment plays a major role in tumour initiation, invasion, and metastasis [77]. Studies on the immune status of POVLs are lacking.

In one study, there was stromal  $\alpha$ -SMA positivity in the stroma of 100% of the OSCC cases and 93% of the OVC cases, but in none of the OVH cases. In contrast, there was stromal

CD34 positivity in 100% of OVH, whereas only 20% of OVC and 11% of OSCC stroma expressed CD34 [32]. The OSCC and OVC groups demonstrated a significant difference in the expression of CD34 and  $\alpha$ -SMA compared to the OVH group ( $P < .0001$ ) [32]. A recent study on fibroblasts in OSCC demonstrated that  $\alpha$ -SMA-positive cancer-associated fibroblasts (CAFs) correlated with a poor clinical outcome ( $p < 0.05$ ) [78]. Akrish et al. showed that  $\alpha$ -SMA expression in CAFs was statistically lower in transformed OSCC from PVLs (p-OSCC) (6%) compared to 40% in conventional OSCCs (c-OSCC) ( $p < 0.0004$ ) [48] and stated that this might explain the slower growth and less invasive behaviour in p-OSCCs compared to conventional OSCC [33].

## Discussion

### Hypothetical overview of POVL progression

The genomic changes identified in POVLs in this study are summarised in **Figure 4**. In keeping with the current histological understanding, POVLs show progressive genetic changes. It appears that OVH and OVC share similar allelic losses present in OED and OSCC. Similarly, POVLs develop chromosomal instability giving rise to aneuploidy at an early stage [15]. The main difference is in the accumulation of these losses. Whilst OED and OSCC progression is characterised by a gradual increase in accumulation of LOH on different chromosomal arms as it progresses through low to high-grade dysplasia, OVH showed frequent losses, including multiple losses, at an early stage. Strikingly, OVH and high-grade dysplasia show similar LOH only differing at 17p [35]. OVH shows an increase in LOH at 9p and 4q during the transition to OVC [35].

OVC has also been considered as the malignant counterpart of OVH, but it is now evident that many OVH cases also transform to OSCC, whilst some cases transform to OVC and then to OSCC [2]. OVH and OVC show fewer CNAs compared to OSCC. The CNAs in OVH and OVC are similar, with more alterations noted in OVC [34]. Also, this study highlighted shared genes

between OVH and OVC. It appears that OVH lesions can progress down one of two pathways: one path to become OVC or the other path to transform to AVH/PVL and OSCC. The intermediary disturbed stage appears to be AVH/PVL, although whether OVC is an end stage, or an intermediary stage remains unclear. OVC has been proven by NGS to be a genetically different carcinoma, contrary to the previous assertion that it is a type of OSCC [79].

In the recent oral cancer genome atlas (CGA), where over 60% of cases were oral cavity cancers, a subgroup of HPV-negative OSCCs with no smoking history, favourable clinical outcomes, and low CNAs was identified. Based on the findings of this review, p-OSCC should show HPV-negative-OSCC genomic profile, better overall prognosis, and short-term survival ( $p=0.03$ ) but not long-term survival ( $p=0.12$ ) [33]. Interestingly, 11q13 amplification noted by Wu et al. [47] corresponding to *CCND1*, *CTTN*, and *FADD* gene area was identified to be co-amplified in this subgroup of HPV-ve OSCCs in the CGA [63]. It is also known that p-OSCC patients are largely non-smokers, which coincides with this new OSCC entity that could represent p-OSCC [63]. This new OSCC category displayed “infrequent CNAs with activating mutations of *HRAS* or *PIK3CA*, coupled with inactivating mutations of *CASP8*, *NOTCH1* and *TP53*” [63]. One subgroup showed loss of function alterations in WNT pathway genes *AJUBA* and *FAT1*, and independently, Samman et al. identified recurrent CNAs in WNT pathway genes in OVH, but no gene mutations were identified in *TP53*, *CDKN2A*, *NOTCH1*, *NOTCH2*, and *FAT1* genes in OVC [79]. Regardless, this new subtype of OSCC appears to show close genomic association with the OVH/OVC/p-OSCC, and this is worthy of further investigation.

At the initial presentation, OVH carries an important genetic signature identifiable by CNAs - prominent amplifications at 7q22, 7q11.2 regions, and deletions at 5q31 and 17p12 regions [34]. AVH/PVL shows 60% overall LOH in a similar manner to severe dysplasia with a striking lower frequency loss at 17p in OVH and OVC compared to OED/OSCC [35]. In addition, 3p21[34 47] and 9p21 [35] have been identified as commonly involved loci in POVLs (**Figure 3**). OVH and PVL demonstrate disturbances in *TP53* and *CDKN2A* gene loci with notable

allelic losses, which have been implicated as important points in the malignant transformation of OVH. *TP53* gene located at 17p13.1 showed significantly lower LOH in OVH compared to OSCC ( $p=0.037$ ). 17p13 CN gain was associated with the transition from OVH to OSCC in Wu et al. study [47]. Wu et al. followed up on two progressive OVH cases, which transformed to OSCC and identified 125 progressive CNAs in OVH which were also present in the transformed OSCC; this indicates that these genomic alterations occurred before the histological changes became apparent [47]. Poh et al. also studied PVL cases, but Samman et al. studied 16 OVH cases which may not have developed the high-risk changes of AVH with malignant potential. Noteworthy, however, is that all the patients in Wu et al. were also betel quid chewers with a skewed sample where other carcinogenic factors would also have played a role. Whilst allelic loss without CNAs is also a possibility, Samman et al. did not identify any of the CN-altered regions reported in the Wu study in their OVH cohort [34].

Overexpression of the tumour suppressor p53 is a frequent alteration in head and neck premalignant lesions and OSCC [80-82]. p53 expression is tightly regulated at several stages [83]. The results of our systematic review suggest that POVLs show changes in the expression of *TP53*, *CDKN2A*, and possibly *ELAVL1* (through copy number amplification at 19p13). *CDKN2A*, located at the 9p21 chromosomal region, is mutated or deleted in many types of cancers, including OSCC [63]. p14 and p16 are cell cycle regulatory proteins, encoded via the use of alternate reading frames from *CDKN2A*, that promote the action of retinoblastoma protein (pRB) and p53, respectively [84]. Hence, both p16 and p14 have important tumour suppressor functions. LOH at 9p21 is seen in OVH, PVL, OVC, and OSCC indicating that *CDKN2A* plays a role in the progression of POVLs and is altered at all stages. OVH shows lower LOH at the 9p21 locus compared to OVC ( $p=0.062$ ), suggesting that there is an increase in LOH in OVC [22]. Although p53 cellular levels were high in POVLs, no *TP53* mutations have been detected in the studies evaluated in our review except for CN gain at 17p13.1 noted by Wu et al. [47]. Therefore, p53 inactivation possibly happens by non-mutational mechanisms.

HPV infection can cause wild-type p53 suppression in cervical and oropharyngeal cancers via

E6 oncoprotein, but this should result in low levels of p53 protein and not high levels as seen in POVLs [85]. Therefore, whether other cellular proteins such as HuR (*ELAVL1*) or viruses affect p53 via a different mechanism independent of inducing p53 degradation, such as epigenetics, would be worthy of exploration.

## **Conclusion**

We propose a possible hypothetical progressive genomic pathway of POVLs that concurs with the biological behaviour of the associated lesions. POVLs present with disturbed genome even at first presentation as OVH characterised by high LOH, low CNAs, and no currently apparent gene mutations. In its second stage as AVH/PVL (after multiple lesions), the genome changes, acquiring more CNAs and mutations in *CDKN2A* and alterations to *ELAVL1* expression. The transition from OVH to OVC involves an increase in LOH at 9p and 4q, whereas the transition from OVH to OSCC involves increases in LOH at 4q and 17p. The interplay between AVH/PVL/OVC is currently unclear since there are shared genomic characteristics between these lesions, with a possibility of OVC being an intermediary lesion rather than the end result. However, at present, the evidence is lacking, and more comprehensive genomic studies are necessary to further understand the enigma of POVL. At present state, POVLs remain an unresolved mystery, and there is hope that this study will offer an insight into the entangled mass of various possible genetic and epigenetic mechanisms that drive the progression of these enticing lesions.

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## Figure captions

**Fig 1: PRISMA flow diagram-** illustrating the process of literature search [10-13].

**Fig 2: Frequency of genomic gain (A) and loss (B) for OVH and OVC, respectively.** Cytogenetic map locations on the x-axis and % frequency of events is shown on the y-axis. The gains are adapted from the frequency karyograms, and the losses are adapted from the GISTIC plots (Adapted from Samman et al. [34]).

**Fig 3: Common LOH profiles in POVL, OVC, and OSCC.** Cytogenetic map locations are shown on the x-axis, and % frequency of events are shown on the y-axis. (Adapted from Poh et al. [35] & Kresty et al. [22]).

**Fig 4: The clinical, histological, and genomic biomarkers in POVL progression. (A)** Microphotographs illustrating the histologic progression of OVH as multiple lesions (PVL) and then to invasive cancer (OSCC) within 2 years (From pathological archives of William Harvey Hospital, Kent).

**(Ai)** 2015- Initial presentation is exophytic with minimum cytological atypia (OVH), **(Aii)** 2016-The lesion is more florid with deeply borrowing rete pattern and basal expansion (AVH). **(Aiii)** 2017-Squamous cell carcinoma shows invasive islands breaking off from the basal epithelium and infiltrating the underlying connective tissue (OSCC).

**(B)** The genomic changes and biomarkers identified in POVLs in this study.

Footnote: The biomarkers and genomic changes noted above are filtered and selected based on the supportive evidence in order to provide a collective summary basis for future studies.

## Tables

**Table 1: Inclusion and exclusion criteria used in the systematic review**

Inclusion criteria	
Population	Patients histologically diagnosed as having OVH/AVH, and patients clinically followed up and diagnosed as having PVL
Intervention	Lesional/tumour DNA and RNA, or molecular markers analysed by WGS, WES, panel sequencing, array CGH, qPCR, ISH, and immunohistochemistry.
Comparators	If available, compared with transformed OVC, transformed OSCC, NOM, and OED.
Outcome	Genetic variations found in lesional DNA - i.e., Copy number variations (CNV), Allelic imbalances, Gene mutations - deletions, insertions, single nucleotide polymorphisms (SNPs), protein alterations, affected functional pathways, Gene expression levels, molecular and immunohistochemical markers.
Study design	Randomised controlled studies, case-control, cohort and cross-sectional studies, case series.
Exclusion criteria	
	Ongoing studies with incomplete results
	Studies on clinicopathological assessment only
	Single case reports
	Studies on only ploidy analysis of OPVLs
	Studies on only HPV viral aetiology of OPVLs

Footnote: WGS - whole genome sequencing, WES - whole exome sequencing, CGH - comparative genomic hybridization, q-PCR - quantitative polymerase chain reaction, ISH - in-situ hybridization, NOM - normal oral mucosa.

**Table 2:** Summary of the characteristics of the studies included in the review-study design and methodology

Study author and year	Study design	Type of POVL	Comparators	Method of investigation
Kresty [22] (2008)	Retrospective Case-control	20 PVL	NOM	PCR,DNA sequencing, IHC
Poh [35] (2001)	Retrospective cohort	25 OVH	NOM,OED, OVC,OSCC	Microsatellite analysis
Samman [34] (2015)	Retrospective cohort	16 OVH	OSCC, NOM	NGS,CN karyograms
Wu [47] (2018)	Prospective Cohort	269 OVH	OSCC	NGS,SNP array
Kannan [24] (1996)	Retrospective case-control	10 PVL	OSCC,NOM	IHC
Gopalakrishnan [23] (1997)	Retrospective Case-control	10 PVL	OSCC,NOM	IHC, PCR, ISH
Fettig [28] (2000)	Retrospective case series	10 PVL	NOM	IHC
Bagan [29] (2008)	Prospective case-control	10 PVL	NOM, OSCC	PCR
Gouvea [26] (2010)	Retrospective cohort	12 PVL	N/A	IHC
Fox [30] (2010)	Case series	9 PVL	N/A	BAC arrays
Gouvea [27] (2013)	Retrospective case control	21 PVL	NOM	IHC, image cytometry
Thennavan [31] (2015)	Retrospective cohort	7 PVL	N/A	IHC
Akrish [33] (2015)	Retrospective cohort	10 PVL	OSCC (non-PVL)	IHC
Paral [32] (2017)	Retrospective cohort	13 PVL/OVH	OSCC, OVC	IHC
Upadhayaya [25] (2018)	Retrospective cohort	20 PVL	N/A	IHC, ISH
Tsai [39] (1999)	Restrospective case-control	10 OVH	SCC,OSMF,NOM	ELISA
Sakurai [41] (2000)	Restrospective cross sectional	18 OVH	OVC	IHC
Chen [42] (2002)	Restrospective cross sectional	20 OVH	OED,OSMF	RT-PCR, IHC
Wu [43] (2002)	Restrospective case-control	4 OVH	OVC,OSCC	IHC
Lai [44] (2006)	Restrospective cross sectional	31 OVH	OVC,OSCC	IHC
Klieb [36] (2007)	Restrospectivecross sectional	28 OVH	OVC	IHC
Hsieh [45] (2010)	Restrospective cross sectional	11 OVH	OED,OSCC,HK, OSMF	IHC
Lin [37] (2011)	Restrospective case control	30 OVH	OVC	IHC
Habiba [46] (2014)	Retrospective cohort	6OVH,25 OVL	OVC	IHC
Sharma [40] (2016)	Restrospective case-control	27 OVH	OVC	IHC
Niranjan [38] (2018)	Restrospective case-control	10 OVH	NOM,OVC, OSCC,OVH	IHC

Footnote: OSMF-oral submucous fibrosis, HK-hyperkeratosis, IHC-immunohistochemistry, ELISA-enzyme-linked immunosorbent assay, BAC-bacterial artificial chromosomes, SNP-single nucleotide polymorphism, ISH-in situ hybridization, RT-PCR-reverse transcription polymerase chain reaction, M-male, F-female

**Table 3:** Characteristics of the study population

	Number of studies	Number of cases	Age range	Mean age	Male:Female ratio
<b>PVL</b>	12	153	33-87	67.4	1:1.6 (M=49, F=80)
<b>OVH</b>	12	505	14-82	53.5	1:2 (M=19, F=38)
<b>AVH</b>	2	27	23-95	71	1:1.2 (M=11, F=14)
<b>Total</b>	26	685	14-95	63.9	1.3:1 (M=179, F=132)

## Figures

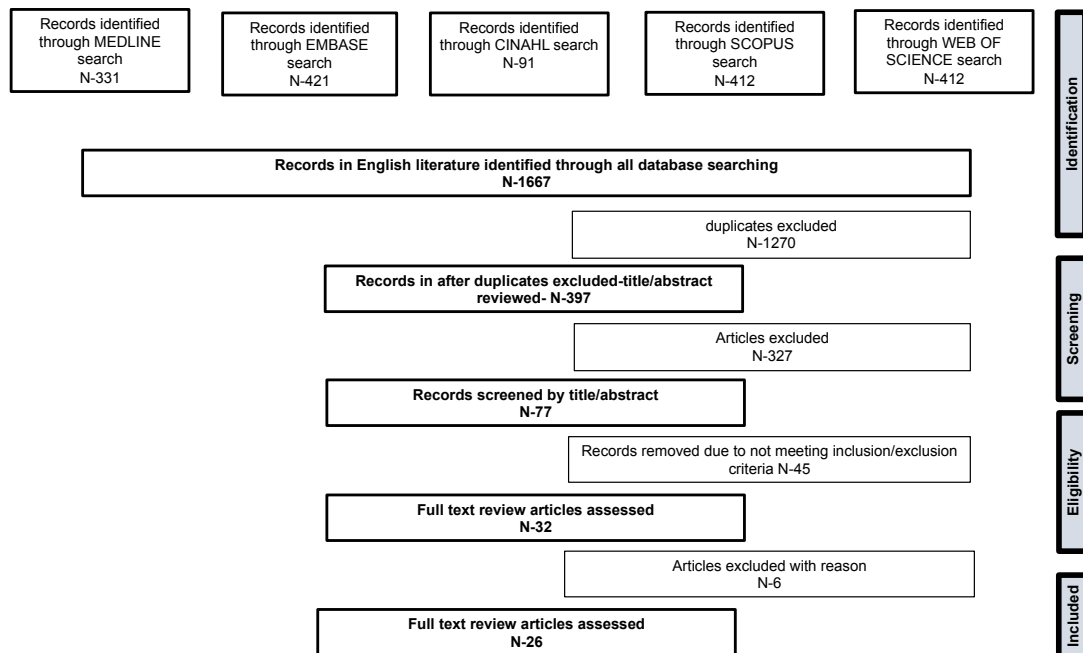


Fig.1

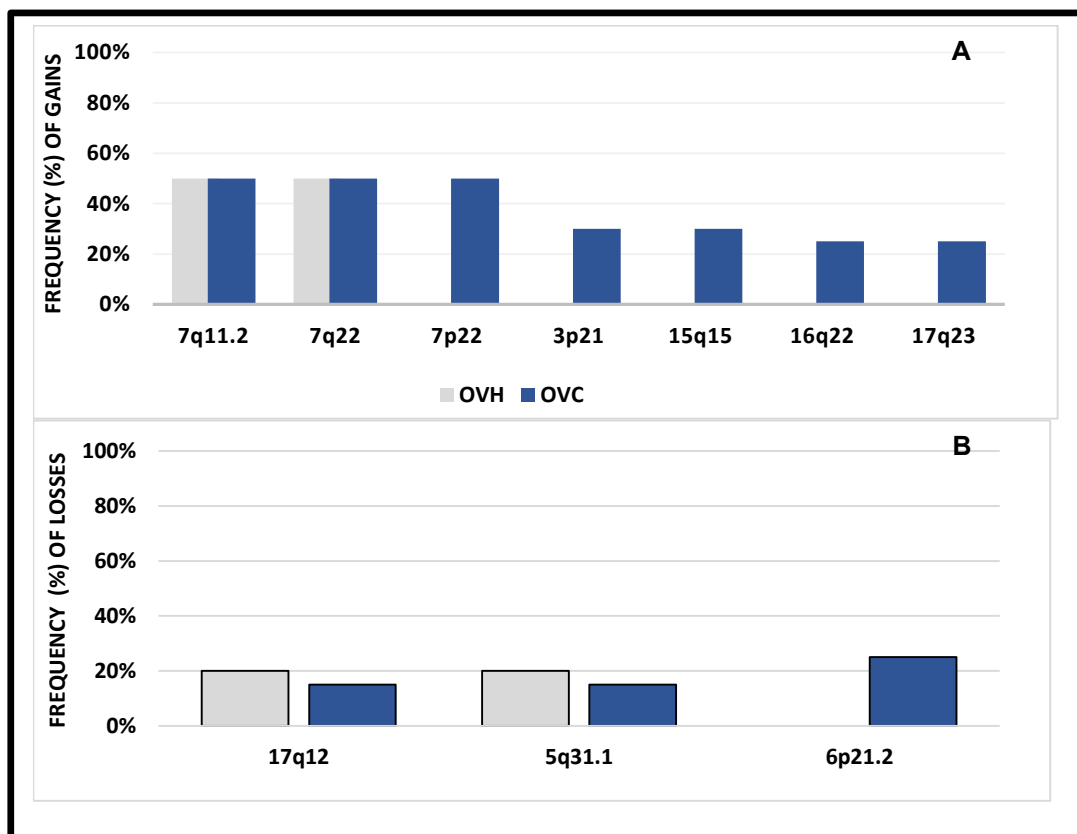


Fig 2

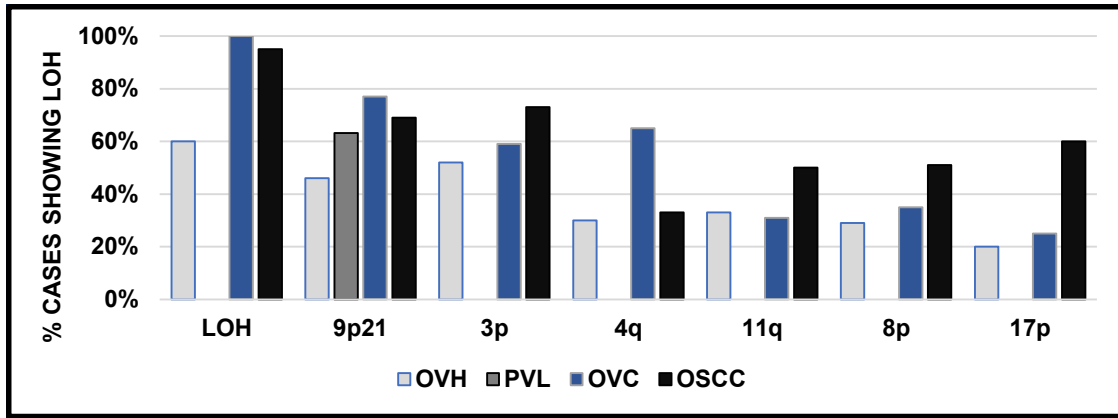


Fig. 3

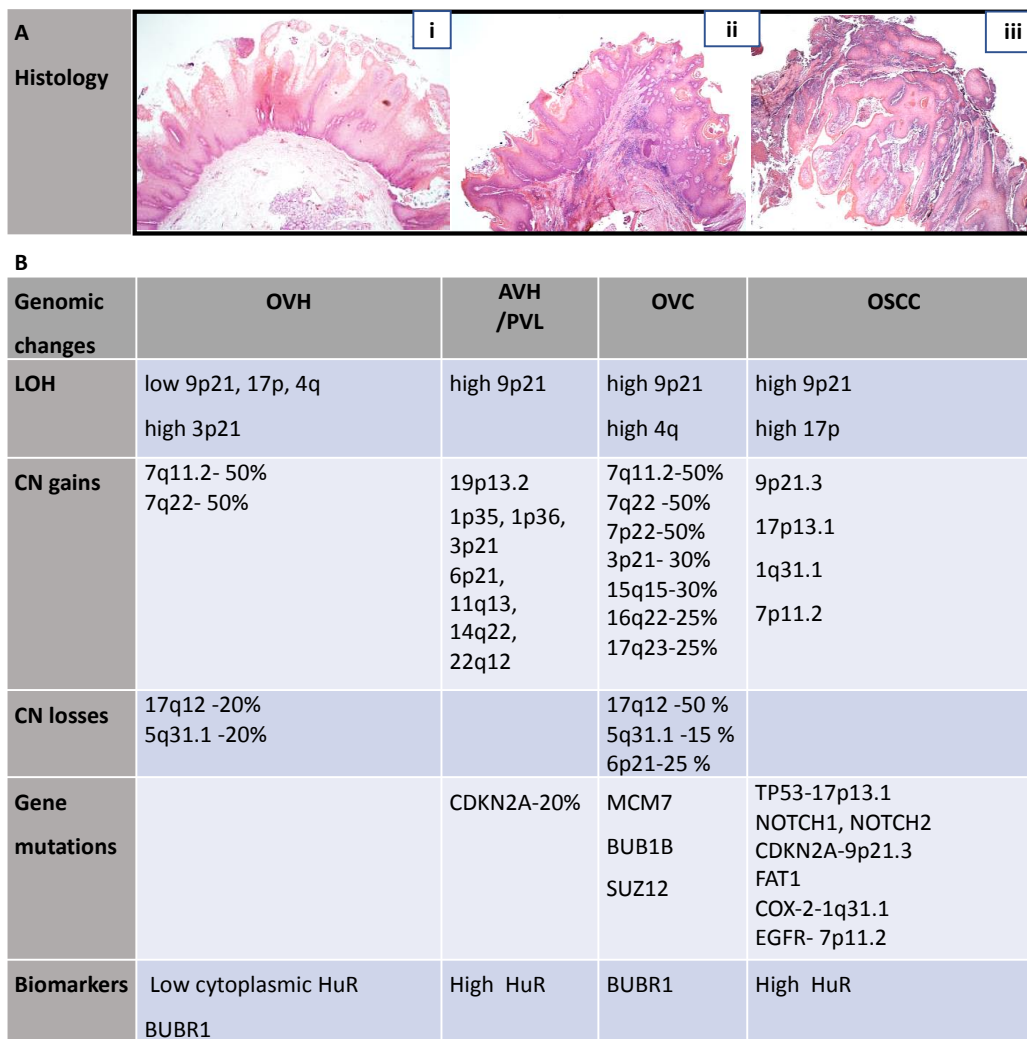


Fig 4

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## Tables

**Table 1:** Inclusion and exclusion criteria used in the systematic review

<b>Inclusion criteria</b>	
Population	Patients histologically diagnosed as having OVH/AVH, and patients clinically followed up and diagnosed as having PVL
Intervention	Lesional/tumour DNA and RNA, or molecular markers analysed by WGS, WES, panel sequencing, array CGH, qPCR, ISH, and immunohistochemistry.
Comparators	If available, compared with transformed OVC, transformed OSCC, NOM, and OED.
Outcome	Genetic variations found in lesional DNA - i.e., Copy number variations (CNV), Allelic imbalances, Gene mutations - deletions, insertions, single nucleotide polymorphisms (SNPs), protein alterations, affected functional pathways, Gene expression levels, molecular and immunohistochemical markers.
Study design	Randomised controlled studies, case-control, cohort and cross-sectional studies, case series.
<b>Exclusion criteria</b>	
	Ongoing studies with incomplete results
	Studies on clinicopathological assessment only
	Single case reports
	Studies on only ploidy analysis of OPVLs
	Studies on only HPV viral aetiology of OPVLs

Footnote: WGS - whole genome sequencing, WES - whole exome sequencing, CGH - comparative genomic hybridization, q-PCR - quantitative polymerase chain reaction, ISH - in-situ hybridization, NOM - normal oral mucosa.

**Table 2:** Summary of the characteristics of the studies included in the review-study design and methodology

Study author and year	Study design	Type of POVL	Comparators	Method of investigation
Kresty [22] (2008)	Retrospective Case-control	20 PVL	NOM	PCR,DNA sequencing, IHC
Poh [35] (2001)	Retrospective cohort	25 OVH	NOM,OED, OVC,OSCC	Microsatellite analysis
Samman [34] (2015)	Retrospective cohort	16 OVH	OSCC, NOM	NGS,CN karyograms
Wu [47] (2018)	Prospective Cohort	269 OVH	OSCC	NGS,SNP array
Kannan [24] (1996)	Retrospective case-control	10 PVL	OSCC,NOM	IHC
Gopalakrishnan [23] (1997)	Retrospective Case-control	10 PVL	OSCC,NOM	IHC, PCR, ISH
Fettig [28] (2000)	Retrospective case series	10 PVL	NOM	IHC
Bagan [29] (2008)	Prospective case-control	10 PVL	NOM, OSCC	PCR
Gouvea [26] (2010)	Retrospective cohort	12 PVL	N/A	IHC
Fox [30] (2010)	Case series	9 PVL	N/A	BAC arrays
Gouvea [27] (2013)	Retrospective case control	21 PVL	NOM	IHC, image cytometry
Thennavan [31] (2015)	Retrospective cohort	7 PVL	N/A	IHC
Akrish [33] (2015)	Retrospective cohort	10 PVL	OSCC (non-PVL)	IHC
Paral [32] (2017)	Retrospective cohort	13 PVL/OVH	OSCC, OVC	IHC
Upadhayaya [25] (2018)	Retrospective cohort	20 PVL	N/A	IHC, ISH
Tsai [39] (1999)	Restrospective case-control	10 OVH	SCC,OSMF,NOM	ELISA
Sakurai [41] (2000)	Restrospective cross sectional	18 OVH	OVC	IHC
Chen [42] (2002)	Restrospective cross sectional	20 OVH	OED,OSMF	RT-PCR, IHC
Wu [43] (2002)	Restrospective case-control	4 OVH	OVC,OSCC	IHC
Lai [44] (2006)	Restrospective cross sectional	31 OVH	OVC,OSCC	IHC
Klieb [36] (2007)	Restrospectivecross sectional	28 OVH	OVC	IHC
Hsieh [45] (2010)	Restrospective cross sectional	11 OVH	OED,OSCC,HK, OSMF	IHC
Lin [37] (2011)	Restrospective case control	30 OVH	OVC	IHC
Habiba [46] (2014)	Retrospective cohort	6OVH,25 OVL	OVC	IHC
Sharma [40] (2016)	Restrospective case-control	27 OVH	OVC	IHC
Niranjan [38] (2018)	Restrospective case-control	10 OVH	NOM,OVC, OSCC,OVH	IHC

Footnote: OSMF-oral submucous fibrosis, HK-hyperkeratosis, IHC-immunohistochemistry, ELISA-enzyme-linked immunosorbent assay, BAC-bacterial artificial chromosomes, SNP-single nucleotide polymorphism, ISH-in situ hybridization, RT-PCR-reverse transcription polymerase chain reaction, M-male, F-female

**Table 3:** Characteristics of the study population

	Number of studies	Number of cases	Age range	Mean age	Male:Female ratio
<b>PVL</b>	12	153	33-87	67.4	1:1.6 (M=49, F=80)
<b>OVH</b>	12	505	14-82	53.5	1:2 (M=19, F=38)
<b>AVH</b>	2	27	23-95	71	1:1.2 (M=11, F=14)
<b>Total</b>	26	685	14-95	63.9	1.3:1 (M=179, F=132)

## Figure captions

**Fig 1: PRISMA flow diagram**- illustrating the process of literature search [10-13].

**Fig 2: Frequency of genomic gain (A) and loss (B) for OVH and OVC, respectively.** Cytogenetic map locations on the x-axis and % frequency of events is shown on the y-axis. The gains are adapted from the frequency karyograms, and the losses are adapted from the GISTIC plots (Adapted from Samman et al. [34]).

**Fig 3: Common LOH profiles in POVL, OVC, and OSCC.** Cytogenetic map locations are shown on the x-axis, and % frequency of events are shown on the y-axis. (Adapted from Poh et al. [35] & Kresty et al. [22]).

**Fig 4: The clinical, histological, and genomic biomarkers in POVL progression. (A)** Microphotographs illustrating the histologic progression of OVH as multiple lesions (PVL) and then to invasive cancer (OSCC) within 2 years (From pathological archives of William Harvey Hospital, Kent).

**(Ai)** 2015- Initial presentation is exophytic with minimum cytological atypia (OVH), **(Aii)** 2016-The lesion is more florid with deeply borrowing rete pattern and basal expansion (AVH). **(Aiii)** 2017-Squamous cell carcinoma shows invasive islands breaking off from the basal epithelium and infiltrating the underlying connective tissue (OSCC).

**(B)** The genomic changes and biomarkers identified in POVLs in this study.

Footnote: The biomarkers and genomic changes noted above are filtered and selected based on the supportive evidence in order to provide a collective summary basis for future studies.

## Figures

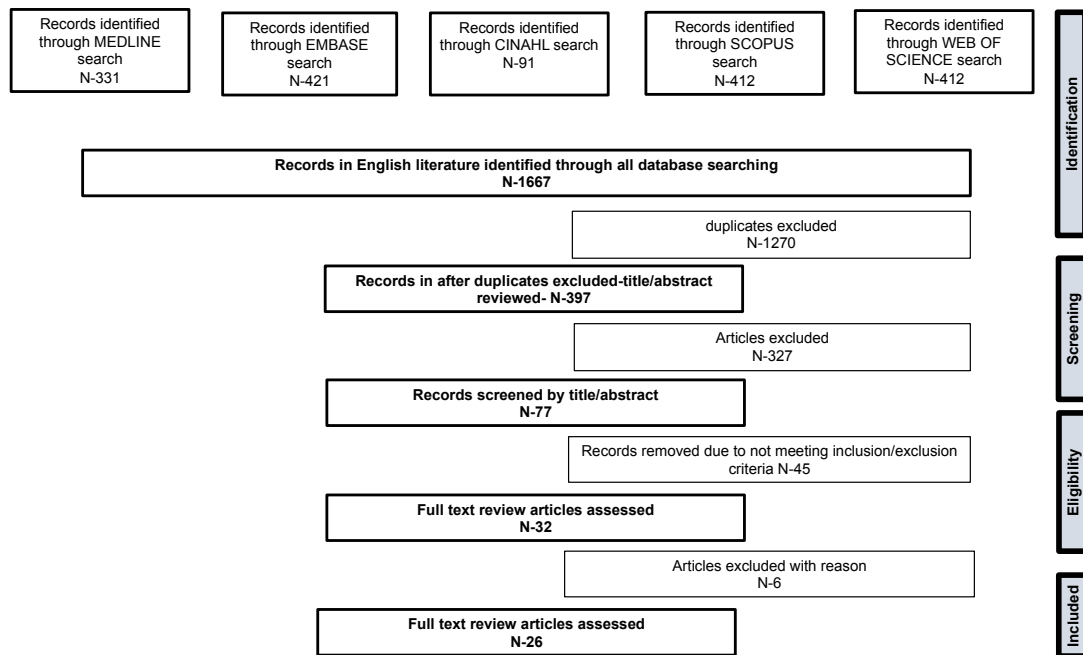


Fig.1

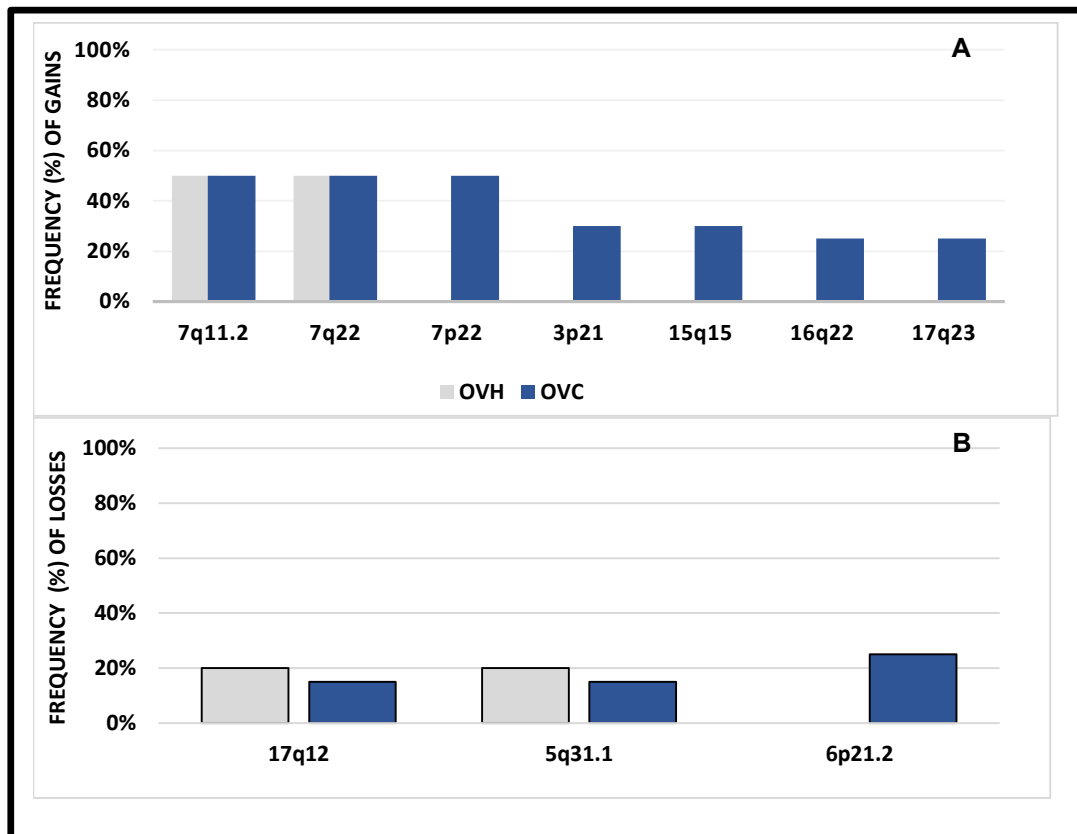


Fig. 2

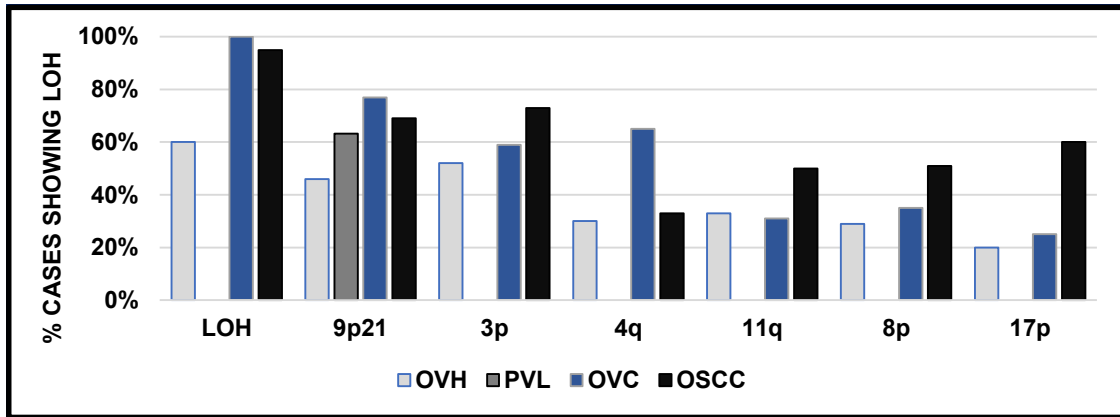
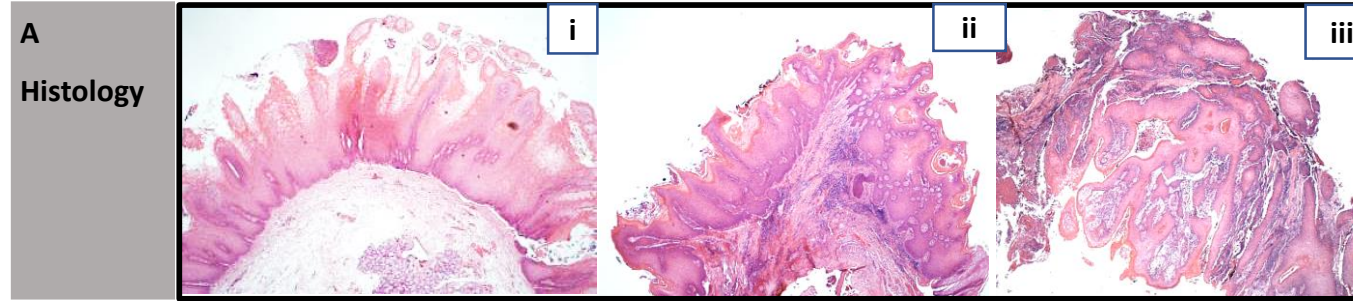


Fig. 3

Figure 4



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**B**

Genomic changes	OVH	AVH /PVL	OVC	OSCC
<b>LOH</b>	low 9p21, 17p, 4q high 3p21	high 9p21	high 9p21 high 4q	high 9p21 high 17p
<b>CN gains</b>	7q11.2- 50% 7q22- 50%	19p13.2 1p35, 1p36, 3p21 6p21, 11q13, 14q22, 22q12	7q11.2-50% 7q22 -50% 7p22-50% 3p21- 30% 15q15-30% 16q22-25% 17q23-25%	9p21.3 17p13.1 1q31.1 7p11.2
<b>CN losses</b>	17q12 -20% 5q31.1 -20%		17q12 -50 % 5q31.1 -15 % 6p21-25 %	
<b>Gene mutations</b>		CDKN2A-20%	MCM7 BUB1B SUZ12	TP53-17p13.1 NOTCH1, NOTCH2 CDKN2A-9p21.3 FAT1 COX-2-1q31.1 EGFR- 7p11.2
<b>Biomarkers</b>	Low cytoplasmic HuR BUBR1	High HuR	BUBR1	High HuR