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REVIEW

Diagnostic Biomarkers in Oral Verrucous Carcinoma: A Systematic Review

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Abstract Oral verrucous carcinoma (OVC), a low-grade variant of oral squamous cell carcinoma (OSCC), is most frequently seen in the oral cavity. No clear etiology has been found for this lesion, but human papilloma virus, chewing betel nuts, and ultraviolet radiation are suggested as probable causes. Differential diagnosis of OVC is challenging for oral pathologists. The aim of this study was to review the molecular-based approaches for differential diagnosis of OVC. An electronic search was conducted in Medline and Scopus from January 2004 to July 2015 limited to English language publications. Published papers on verrucous carcinoma (VC) were found according to the inclusion and exclusion criteria and analyzed qualitatively. Data extraction were performed according to PRISMA statement. A total of 423 articles were reviewed; out of which, 26 articles completely fulfilled the inclusion criteria. Most of the included studies investigated proliferative and apoptotic biomarkers such as p53 and Ki67. No definite conclusion was drawn for cytoskeletal biomarkers due to variability of factors and lack of significant expression. However, it seems that cytokeratin10 (CK 10) can be useful for differentiation of OVC and benign squamous lesions. Among cell surface and extracellular matrix biomarkers tissue biomarkers, matrix metalloproteinase (MMP)-2, −9, CD31 and CD68 seem to be useful for differentiation of OVC and OSCC and glucose transporter-1 (GLUT-1) can help in differentiation of OVC from oral epithelial dysplasia. Differences among OVC, OSCC and normal epithelium in expression profiles of the investigated biomarkers help in their differential diagnosis; although, clinicohistopathological similarities among verrucous hyperplasia, noninvasive OVC and invasive well-differentiated OSCC make the diagnosis difficult. Further studies are required to better differentiate these oral lesions.

Keywords Biomarker · Verrucous carcinoma · Oral carcinoma

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Introduction

As the sixth most common cancer in the world, head and neck squamous cell carcinoma (SCC) has an incidence of nearly 600,000 cases per year; while the 5-year survival rate is almost 50% [1]. A rare and low-grade variant of SCC is VC, which is also known as "Ackermann's tumor" [2]. The most frequent site of involvement for VC is the oral cavity and therefore called OVC. Although there is no clear etiology for VC, some believe the human papilloma virus to be the cause [3]. Chewing betel nuts [4], chronic use of tobacco and ultraviolet radiation are some other suggested causes [5, 6]. The most common presentation of VC is an exophytic lesion with pebbly mamillated and fungating surface, although not all the VC cases appear as typical warty exophytic lesions [7, 8]. Some other OVC characteristics include slow growth and localized invasion with no metastases; although it can destroy adjacent tissues when it grows into a large lesion [9]. It has been reported that the most common sites of oral mucosal involvement are the buccal mucosa, gingiva and tongue [10]. Local lymphatic metastases and

recurrence are also seen in some cases [11]. Histological characteristics of VC include parakeratotic and non-dysplastic epithelium, high order of epithelium differentiation with barely any mitotic activity and pleomorphism. Without any interruption of the basement membrane, deep bulbous epithelial ridges push into the underlying connective tissue [10, 12, 13]. As previously described by Shear and Pindborg, verrucous hyperplasia (VH) resembles a premalignant lesion, and apparently OVC and VH share the same clinical and pathological features [14]. Moreover, some foci of SCC may be observed in 20% of VC cases, making it a hybrid tumor and conferring a metastatic potential to it [15]. Thus, the frequency of initial misdiagnosis is high and histopathological diagnosis of VC is difficult. Unfortunately, the clinico-histological similarities may lead to inter-observer and intra-observer misdiagnosis, and therefore an accurate differentiation should be done by pathologists. Like OSCC, the treatment of choice for VC is surgery but a neck dissection does not seem necessary [16, 17]. Although it has been stated that radiotherapy has the potential to cause anaplastic transformation [18–20], radiotherapy is used with or without chemotherapy in OSCC [21]. In addition, pure VC is treated more conservatively than conventional SCC; hybrid tumors should be treated similar to staged conventional SCC [22, 23]. In the current study, we review molecular-based approaches for differential diagnosis of VC.

Methods

An electronic search was conducted in Medline, EMBASE and Scopus from January 2004 to July 2015 limited to English language publications with available full texts. Published papers on VC were found using the following keywords alone or ensemble: squamous cell carcinoma, oral, verrucous carcinoma, verruciform, immunohistochemistry and markers. All studies on OVC were reviewed. There were studies regarding differential diagnosis based on clinicohistopathological features and molecular examinations, which investigated new molecular and immunohistochemical tests for OVC and were included. Studies that compared OVC, verrucous hyperplasia, verruciform OSCC and normal mucosa were also included. Experiments, which did not investigate cellular and molecular responses or only compared clinicohistopathological features were excluded (Fig. 1).

Initial paper selection was done by assessing the titles and abstracts of the selected papers. The full texts of the potentially suitable articles were obtained for final assessment according to the inclusion and exclusion criteria. Figure 1 demonstrated the flow chart diagram of the present study selection according to PRISMA guidelines [24].

Results

A total of 423 articles were reviewed. Sixty-five articles were included as relevant for the purpose of this systematic review (Fig. 1). Following the initial screening of titles and abstracts and the final screening of full texts, 26 articles completely fulfilled the inclusion criteria for this study. The following results were obtained:

Cell Surface Proteins

Five studies used cell surface biomarkers [25–29]. Five of them used immunohistochemistry and the remaining one used quantitative real-time polymerase chain reaction (Q-RT PCR). Biomarkers such as CK-10, −13, −14, −16, and −20 were evaluated in the studies (Table 1).

In the study of Vidiya et al., the expression level of GLUT-1 was investigated in OED, OSCC and VC, which was seen in more than 50% of VC cases and 25–50% of OSCC cases. The expression percentage positivity increased progressively from NOM to OED, to OSCC and VC ($P < 0.001$). The expression of GLUT-1 was different in mild, moderate and severe OED and was the highest in severe OED [25].

Gao et al. showed the expression level of CK20 to be 100% in OVC cases, 90.6% in OSCC cases and 55.5% in benign squamous lesion (BSL) cases ($p < 0.001$); also, there were no significant differences in expression levels of BSL and dysplastic mucosa adjacent to carcinoma ($p > 0.1$) [28].

In 2010, El-Rouby et al., immunohistochemically examined the distribution of tumor associated macrophages in OSCC and OVC. The area percentage of CD68 immunoreactivity and microvessel density (MVD) were significantly lower in OVC compared with different grades of OSCC ($p = 0.0009$ and $p = 0.0045$, respectively). CD31 immunostain was identified in the stroma of all studied specimens. Although an increase in MVD was associated with high-grade malignancy of SCC, the difference in the MVD between grades was not statistically significant ($F = 1.8859$, $p = 1.765$). Also, MVD was significantly lower in OVC compared with OSCC [27].

Cytoskeletal Proteins

Four studies used cytoskeletal biomarkers [28–31]. The expression of biomarkers such as a-smooth muscle actin (SMA), cytokeratin (CK) 10, 13, 14, 20 and β-catenin were evaluated therein. All studies used immunohistochemistry approach (Table 2).

Paral et al. evaluated the use of CD34 and a-SMA to distinguish VC and VH. The results suggested that a-SMA is present in 93% of VC cases and 0% of VH cases

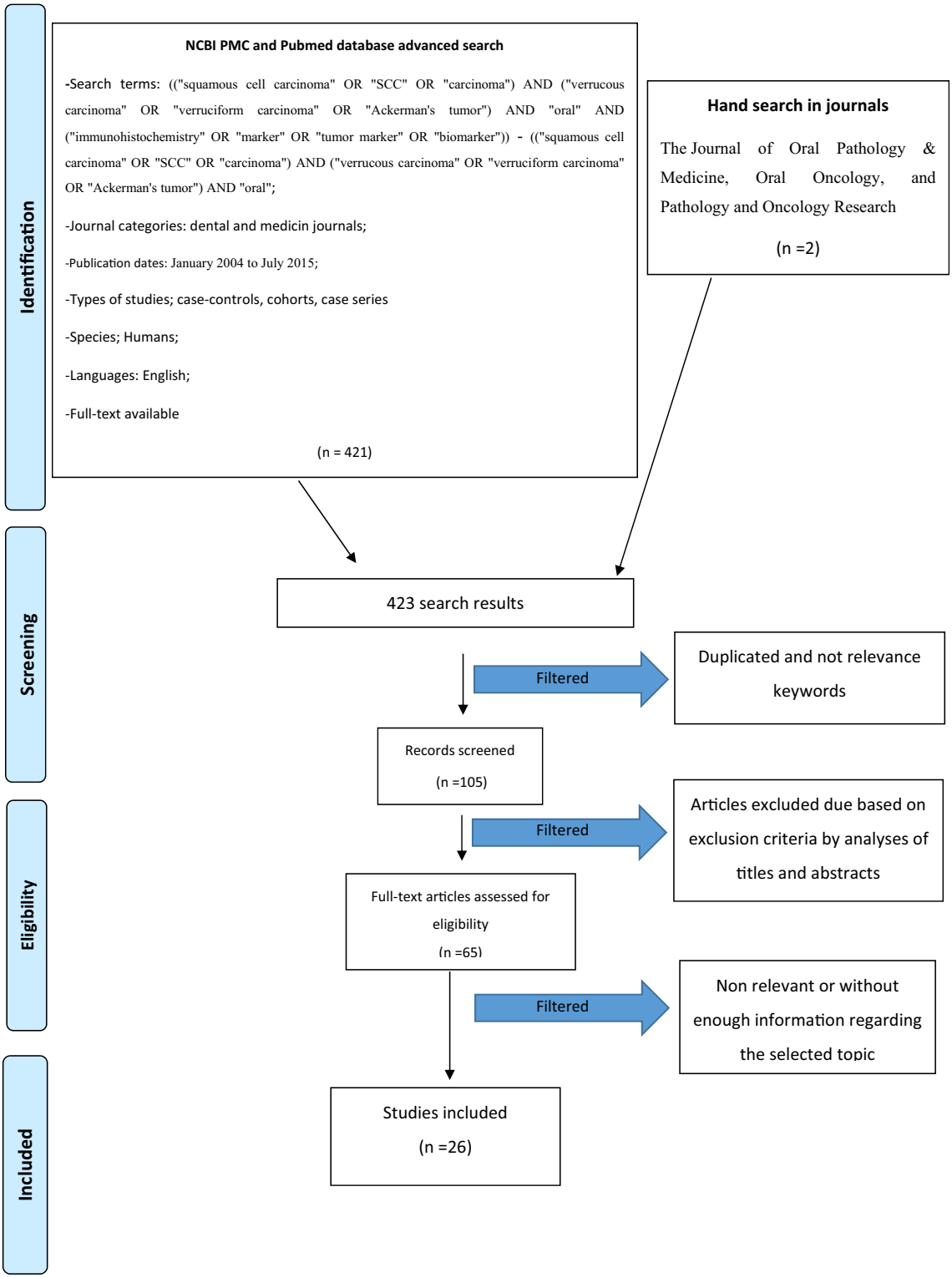

Fig. 1 PRISMA flow diagram of search strategy used in this study

($p < 0.001$). Also, CD34 was observed in 100% of VH cases and 20% of VC cases ($p < 0.001$) [29].

Laxmidevi et al. evaluated the expression of β-catenin in VC and different grades of OSCC. The results indicated significant correlation of β-catenin expression between moderately differentiated SCC (MDSCC) and poorly differentiated SCC (PDSCC) ($p < 0.5$) and well differentiated SCC (WDSCC) and PDSCC; but there were no significant differences between OSCC and VC ($p = 0.3871$) [30]. The expression patterns of CK-10, −13, −14 and −16 in OVC and oral

Table 1 Cell surface proteins

Authors & year	Approach	Biomarkers	Results
C. Angadi V et al. [25], 2015	Imm.	GLUT-1	GLUT-1 Intensity: OVC and OED: $p = 0.004$ OSCC and OED: $p = 0.004$ Mild OED and Severe OED: $p = 0.009$ GLUT-1 Percentage**:** NOM and OVC: $p < 0.001$ NOM and OSCC: $p < 0.001$ NOM and OED: $p < 0.001$ Mild OED to Severe OED: $p < 0.001$ WDSCC and PDSCC: $p = 0.015$
Wang Y-H et al. [26], 2014	Q-RT-PCR	Differentially Expressed Genes (more than 2-fold)	Gene Expression: Between OVC and OSCC: ADAMTS12*, Col. IV A1, Col. IV A2, INHBA*, MMP1, SERPINE1*, TGFB1 up-regulated and HLF*down-regulated.
El-Rouby DH et al. [27], 2010	Imm.	CD68 and CD31	CD68: OVC and OSCC: $P = 0.0009$ WDSCC, MDSCC and PDSCC: $P = 0.0733$ CD31: OVC and OSCC: $p = 0.0045$ WDSCC, MDSCC and PDSCC: $p = 1.765$
Gao H et al. [28], 2009	Imm.	CK20, CD10 and CD 34	Expression Levels: CD10: OVC and BSL: $p = 0.25$ OSCC and BSL: $p = 0.875$ OVC & OSCC and BSL: $p = 0.26$ CD34: OVC and BSL: $p < 0.001$ OSCC and BSL: $p < 0.001$ OVC & OSCC and BSL: $p < 0.001$

BSL Benign Squamous Lesion, *CD* Cluster Differentiation, *CK* Cytokeratin, *GLUT* Glucose Transporter, *Imm* Immunohistochemistry, *MDSCC* Moderately Differentiated Squamous Cell Carcinoma, *OVC* Oral Verrucous Carcinoma, *PDSCC* Poorly Differentiated Squamous Cell Carcinoma, *QRT-PCR* Quantitative Real-Time Polymerase Chain Reaction, *OSCC* Squamous Cell Carcinoma, *SED* Severe Epithelial Dysplasia, *TGF* Transforming Growth Factor, *WDSCC* Well Differentiated Squamous Cell Carcinoma

*Gene name

squamous papilloma (OSP) were investigated in a study by Oliviera et al. The results indicated that in OVC, CK-10 was expressed in suprabasal to superficial layers and CK-13 was detected in prickle cells and superficial cells in most cases; also all the cell layers of OVC were positive for CK-14. Eventually, CK-16 was observed in suprabasal to the superficial layer; whereas, CK10 was observed in suprabasal to superficial cells of OSP, CK-13 was observed in suprabasal to superficial cells of OSP and in contrast to OVC, only basal and suprabasal layers of OSP were more pronounced for CK-14. The majority of cases in OSP showed only superficial reactive cells to CK-16 [31].

Gao et al. described sequential changes in CD10+ and CD34+ stromal cells (SCs) during the transition of oral lesions from benign to malignant and suggested that the mean number of CD34+ SCs was significantly lower in VC (57.36) and SCC (33.81) than BSL (351.56, $p < 0.001$) and that the three tumor types had the same staining level and number of CD10+ SCs [28].

Extracellular Matrix Proteins

Five studies investigated extracellular matrix biomarkers [26, 32–35]. These articles assessed the expression of Col. IV and Ln-332 c2, VEGF, MMP-2 and -9, NQO1 and SOD (Table 3).

Table 2 Cytoskeletal proteins

Authors & year	Approach	Biomarkers	Results
Paral KM et al. [29], 2014	Imm.	a-SMA + CD34	a-SMA Positivity: OVC and VH: $p < 0.001$ OSCC and VH: $p < 0.001$ OVC and OSCC: $p = 0.91$ CD34: OVC and VH: $p < 0.001$ OSCC and VH: $p < 0.001$ OVC and OSCC: $p = 0.41$
Laxmidevi LB et al. [32], 2010	Imm.	β-catenin	WDSCC and MDSCC: $p = 0.0494$ WDSCC and PDSCC: $p = 0.0003$ MDSCC and PDSCC: $p = 0.4274$ OSCC and OVC: $p = 0.3871$
Gao H et al. [28], 2009	Imm.	CK20, CD10 and CD 34	Expression Levels: CK20: BSL and OVC: $P < 0.001$ BSL and OSCC: $P < 0.001$ BSL and dysplastic mucosa adjacent to carcinoma: $P > 0.1$
Oliveira M et al. [33], 2005	Imm.	CK 10, 13, 14 and 16	OVC: CK 10: Basal–suprabasal:0* / Prickle–superficial:0 / Suprabasal–superficial:8 / Superficial: 0 /All layers:0 CK 13: Basal–suprabasal:0 / Prickle–superficial:6 / Suprabasal–superficial:2 / Superficial: 0 / All layers:0 CK 14: Basal–suprabasal:0 / Prickle–superficial:0 / Suprabasal–superficial:0 / Superficial: 0 / All layers:8 CK 16: Basal–suprabasal:0 / Prickle–superficial:0 / Suprabasal–superficial:7 / Superficial: 0 / All layers:1 OSP: CK 10: Basal–suprabasal:0 / Prickle–superficial:1 / Suprabasal–superficial:2 / Superficial: 5 / All layers:0 CK 13: Basal–suprabasal:0 / Prickle–superficial:0 / Suprabasal–superficial:8 / Superficial: 0 / All layers:0 CK 14: Basal–suprabasal:7 / Prickle–superficial:0 / Suprabasal–superficial:0 / Superficial: 0 / All layers:1 CK 16: Basal–suprabasal:0 / Prickle–superficial:2 / Suprabasal–superficial:1 / Superficial: 5 / All layers:0

BSL Benign Squamous Lesion, *CD* Cluster Differentiation, *Col*. Collagen, *CK* Cytokeratin, *Imm*. Immunohistochemistry, *OSCC* Oral Squamous Cell Carcinoma, *OVC* Oral Verrucous Carcinoma, *PDSCC* Poorly Differentiated Squamous Cell, *SED* Severe Epithelial Dysplasia, *SMA* Smooth Muscle Actin, *VH* Verrucous Hyperplasia, *WDSCC* Well Differentiated Squamous Cell Carcinoma

*Number of cases

Arduino et al. investigated the immunohistochemical expression of laminin, laminin-5, collagen IV and fibronectin in VC, severe epithelial dysplasia (SED) and SCC. The staining pattern of laminin was less defined in SCC compared with SED ($p = 0.041$) and VC ($p = 0.017$); although this difference was not significant. The basement membrane laminin staining was more discontinuous in SED than VC ($p = 0.002$), and the same results were found for type IV collagen ($p = 0.025$) and fibronectin ($p = 0.03$). Type IV collagen stained more strongly and was more defined in VC than in SED ($p = 0.048$); but, between VC and SCC and between SCC and SED, the staining intensity did not differ [34].

In 2011, Zargaran et al., immunohistochemically assessed type IV collagen expression in well differentiated OSCC and OVC. In their study, three groups of epithelial hyperplasia with no dysplasia (group A), OVC (group B) and well differentiated OSCC (group C) were compared. The results indicated significant differences in type IV collagen staining patterns among the three groups ($p = 0.000$). Also, there were significant differences between groups A and B ($p = 0.000$) and A

Table 3 Extracellular matrix proteins

Authors & year	Approach	Biomarkers	Results
Wang Y-H et al. [26], 2014	Q-RT-PCR	Differentially Expressed Genes (more than 2-fold)	Genes Expression: Between OVC and OSCC: ADAMTS12*, Col. IV A1, Col. IV A2, INHBA*, MMP1, SERPINE1*, TGFB1 up-regulated and HLF* down-regulated.
Mohtasham N et al. [34], 2013	Imm.	p53, Ki-67, MMP-2 and MMP-9	MMP-2: High grade OSCC and OVC: $p < 0.001$ High and Low grade OSCC and OVC: $p < 0.001$ Low grade OSCC and OVC: $p = 0.3$ MMP-9: High grade OSCC and OVC: $p < 0.001$ High and Low grade OSCC and OVC: $p < 0.001$ Low grade OSCC and OVC: $p < 0.001$
Zargaran M et al. [30], 2011	Imm.	Col. IV and Ln-332 c2	Col. IV: EH and OVC: $p = 0.000$ EH and OSCC: $p = 0.000$ OVC and OSCC: $p = 1$ Ln-332 c2: OVC and OSCC: $p = 0.000$
Ray JG et al. [35], 2011	Imm.	VEGF, MMP-2 and -9, NQO1 and SOD.	Over Expression of All the Proteins in Both OVC and OSCC.
Arduino PG et al. [31], 2010	Imm.	Col. IV and Ln-332 c2	Col. IV: OSCC and OVC: $p > 0.05$ OSCC and SED: $p > 0.05$ OVC and SED: $p = 0.048$ Ln-332 c2: OSCC and OVC: $p = 0.017$ OSCC and SED: $p = 0.041$ OVC and SED: $p > 0.05$

BSL Benign Squamous Lesion, *CD* Cluster Differentiation, *Col*. Collagen, *GLUT* Glucose Transporter, *Imm*. Immunohistochemistry, *Ln* Laminin, *MMP* Matrix Metalloproteinase, *NOM* Normal Oral Mucosa, *NQO* Nicotinamide Adenine Dinucleotide Phosphate Quinone Oxidoreductase, *OED* Oral Epithelial Dysplasia, *OSCC* Oral Squamous Cell Carcinoma, *OVC* Oral Verrucous Carcinoma, *PDSCC* Poorly Differentiated Squamous Cell, *Q-RT-PCR* Quantitative Real-Time Polymerase Chain Reaction, *SED* Severe Epithelial Dysplasia, *SMA* Smooth Muscle Actin, *SOD* Superoxide Dismutase, *VEGF* Vascular Endothelial Growth Factor, *VH* Verrucous Hyperplasia, *WDSCC* Well Differentiated Squamous Cell Carcinoma

*Gene name

and C ($p = 0.000$). No significant differences were seen in the staining pattern between groups B and C ($p = 1$) [33]. Moreover, they immunohistochemically assessed laminin-332 c2 (Ln-332 c2) chain expression in well-differentiated OSCC, OVC and epithelial hyperplasia with no dysplasia. Ln-332 c2 chain expression was detected only in OSCC and OVC groups ($p = 0.000$) [33].Arduino et al. investigated the immunohistochemical expression of laminin and collagen IV in VC, SED, and SCC. Their results showed that laminin was less intensive in SCC compared with SED and VC; and collagen IV expression increased in VC compared to SED [34].

Mohtasham et al. sought the expression of MMP-2 and MMP-9 in SCC and VC cases. Significant differences were seen in the expression of MMP-9 between all grades of OSCC and OVC ($p < 0.001$). Also MMP-2 expression levels were significantly higher in high grade OSCC than OVC ($p < 0.001$). The results also suggested that MMP-9 is one of the most reliable factors for invasive SCC grading [32]. Ray et al. attempted to differentiate OVC from OSCC by studying the expression patterns of VEGF, MMP 2 and 9, SOD 2 and NQO1. The results indicated overexpression of all the proteins in both OVC and OSCC. They stated that VEGF and MMP-9 may serve as two promising markers for differentiating OVC from OSCC. They also indicated over-expression of nicotinamide adenine dinucleotide phosphate quinine oxidoreductase (NQO1) and superoxide dismutase (SOD) in both OVC and OSCC [35].

Table 4 Proliferative and apoptotic biomarkers

Authors & year	Approach	Biomarkers	Results
Manar Samman et al. [50], 2015	PCR	57 OVC and Exome and RNA	36 Protein-coding Genes Expressed Significantly Different in OVC and OSCC ($p < 0.01$).
Saumyaranjan Mallick et al. [49], 2014	Imm.	Ki-67(MIB-1) and CD34	EPI*: OVC and VH: $p = 0.79$ OVC and OSCC: $p = 0.0001$ MVD**: OVC and VH: $p = 0.7$ OVC and OSCC: $p = 0.001$
Wang Y-H et al. [26], 2014	MH Q-RT-PCR	Differentially Expressed Genes (more than 2-fold)	Gene Expression: OVC and OSCC: ADAMTS12, COL4A1, COL4A2, INHBA, MMP1, SERPINE1, TGFB1 up-regulated and HLF down-regulated.
Patil GB et al. [48], 2013	Imm.	Cyclin B1	WDSCC and MDSCC: $p = 0.351$ WDSCC and PDSCC: $p = 0.0001$ MDSCC and PDSCC: $p = 0.0048$ COSCC and OVC: $p = 0.0065$ WDSCC and OVC: $p = 0.06542$ MDSCC and OVC: $p = 0.0641$ PDSCC and OVC: $p = 0.0001$
Mohtasham N et al. [34], 2013	Imm.	p53, Ki-67, MMP 2 and MMP 9	P53: High grade OSCC and OVC: $p < 0.0001$ High and Low grade OSCC and OVC: $p < 0.001$ Low grade OSCC and OVC: $p < 0.0001$ Ki-67: High grade OSCC and OVC: $p = 0.9$ High and low grade OSCC and OVC: $p = 0.5$ Low grade OSCC and OVC: $p = 0.1$
Odar K et al. [47], 2012	RT-PCR + Imm.	PTE miR-21, miR-31, miR-203, miR-125a-5p and miR125b, PTEN and p63	miR-31: OVC and NOM: $p < 0.001$ miR-125a-5p: OVC and WDSCC & MDSCC: $p < 0.001$ miR-203: OVC and PDSCC: $p < 0.001$ miR-203 to miR-125b ratio: OVC and NOM: $p < 0.001$ OVC and WDSCC & MDSCC: $p < 0.001$ miR-203 to miR-21 ratio: OVC and PDSCC: $p < 0.001$ PTEN: OVC and OSCC: $p = 0.001$ OVC and NOM: $p = 0.001$ P63: OVC and OSCC: $P < 0.001$ OVC and NOM: $P < 0.001$
Terada T et al. [46], 2012	Imm.	p53 and Ki-67	p53 protein Was Expressed in Both VC and SCC, Though the Expression in OSCC Was More and Broad than that in OVC. Ki- 67: OSCC = 64% OVC = 12%
Zargaran M et al. [45], 2012	Imm.	Ki-67	OVC and MOSCC: $p = 0.85$ MOSCC and WDSCC: $p = 0.83$ OVC and WDSCC: p = $p = 0.3$
Terada T et al. [44], 2011	Imm.	P53 and Ki-67	1. Positive p53 Protein in all Ten Cases, With Location Accentuated Near the Basal Cells and Microinvasive Parts. 2. Ki-67 Positive Cells Were also Seen Mainly among the Basal Cells in Microinvasive Parts, and the Labeling Index Ranged from 12 to 21%.
de Spíndula-Filho JV et al. [43], 2011	Imm.	PCNA, Ki-67 and cyclin B1.	PCNA: OSCC and OVC: $p < 0.05$ OSCC and NOM: $p < 0.05$ Ki-67: OSCC and OVC: $p > 0.05$ OSCC and NOM: $p < 0.05$ Cyclin B1: OSCC and OVC: $p < 0.05$ OSCC and NOM: $p < 0.05$
Ray JG et al. [35], 2011	Imm.	VEGF, MMP-2 and -9, NQO1 and SOD.	Over Expression of All the Proteins in Both OVC and OSCC.
Quan H et al. [42], 2011	Imm.	αB-crystallin, AC-3	αB-crystallin: OVC and OSCC: $p = 0.044$ OVC and NOM: $p = 0.10$ OSCC and NOM: $p = 0.000$ AC-3: OVC and OSCC: $p = 0.202$ OVC and NOM: $p = 0.000$ OSCC and NOM: $p = 0.040$
Wang Y et al. [41], 2011	2-DE + MALDI-TOF	36 proteins	2-DE: OVC: Ten Protein Spots Showed Significant Over Expression. OSCC: Ten Protein Spots Showed Significant Over Expression. MALDI-TOF analysis:

Table 4 (continued)

Authors & year	Approach	Biomarkers	Results
Pentenero M et al. [40], 2011	hr DNA-FCM	Chromosomal instability***	OVC: Zinc Finger Protein 77 showed 33.9% Coverage. OSCC: Phospholipase A2 Inhibitory Protein 64.3% Coverage. DNA-diploid & DNA-aneuploid: OVC and OSCC: $p = 0.649$ One aneuploid subline & Two or more aneuploid sublines: OVC and OSCC: $p = 0.163$
Lin HP et al. [39], 2010	Imm.	p53, MDM2, p21, HSP 70 and HPV 16/18 E6	p53: OVC and OVH: $p = 0.575$ MDM2: OVC and OVH: $p = 0.416$ p21: OVC and OVH: $p = 0.053$ HSP 70: OVC and OVH: $p = 0.252$ HPV 16/18 E6: OVC and OVH: $p = 0.769$
Adegboyega PA et al. [38], 2005	Imm.	p16, p21,p53, Ki-67 and RBGP	p16: OVC, OSCC and Acanthosis: $p = 0.01182$ p21: OVC, OSCC and Acanthosis: $p = 0.00064$ p53: OVC, OSCC and Acanthosis: $p = 0.00000$ Ki-67: OVC, OSCC and Acanthosis: $p = 0.00000$ RBGP: OVC, OSCC and Acanthosis: $p = 0.00000$
Tran TN et al. [37], 2005	MSP	LOH of 3p21 and 9p21, Hypermethylation of p16INK4a and RASSF1A	LOH: There Was no Significant Correlation Between LOH at 3p, 9p and the Pathological Grading or Stage ($p = 0.218/p = 0.711$ for 3p and $p = 0.1/p = 0.893$ for 9p). p16INK4a****: OVC: $p = 0.5$ OSCC: $p = 0.319$ RASSF1A****: OVC: $p = 1$ OSCC: $p = 1$
Nishikawa T et al. [36], 2005	Imm.	H3 mRNA, p53, Cyclin D1 and B1	Cyclin B1: OVC: 69% SP: 85% HK: 75% Cyclin D1: OVC: 65% SP: 78.6% HK: Limited to the parabasal layer. P53: OVC: 50% SP: 86.4% HK: 25% H3 mRNA: OVC: 90.9% SP: 88.9% HK: 66.7%

AC-3 Activated Caspase-3, *COL* Collagen, *DE* Dimensional Electrophoresis, *DNA* Deoxyribonucleic Acid, *EHWD* Epithelial Hyperplasia Without Dysplasia, *EPI* Endothelial Proliferative Index, *Imm* Immunohistochemistry, *HPV* Human Papilloma Virus, *OVC* Oral Verrucous Carcinoma, *MDM*, *MDSCC* Moderately Differentiated Squamous Cell Carcinoma, *MMP* Matrix Metalloproteinase, *MSP* Methylation-specific Polymerase Chain Reaction, *MVD* Microvascular Density, *NQO* Nicotinamide Adenine Dinucleotide Phosphate Quinone Oxidoreductase, *PCNA*, *PDSCC* Poorly Differentiated Squamous Cell Carcinoma, *QRT-PCR* Quantitative Real-Time Polymerase Chain Reaction, *RBGP* Retinoblastoma Gene Product, *SCC* Squamous Cell Carcinoma, *SOD* Superoxide Dismutase, *SP* Squamous Papilloma, *VC* Verrucous Carcinoma, *VEGF* Vascular Endothelial Growth Factor, *VH* Verrucous Hyperplasia, *WDSCC* Well Differentiated Squamous Cell Carcinoma

*EPI: Endothelial proliferative index measured by Ki67 counterstained with Periodic Acid Schiff staining to estimate angiogenesis

**MVD: Microvascular density measured by CD34 immunostaining to estimate angiogenesis

*** Chromosomal instability

**** Tumor suppressor genes known as p16INK4a (located in 9p21) and RASSF1A (located in 3p21.3)

Proliferative and Apoptotic Biomarkers

Eighteen studies used proliferative and apoptotic biomarkers [26, 32, 35–50] namely Ki-67, Cyclin B1, Cyclin D1, αB-crystalline, P16, P21 and P53 (Table 4). Most of the studies used immunohistochemistry. Three studies used PCR. One study used exome sequencing and RNA sequencing. One study used methylation-specific polymerase chain reaction (MSP). In

2015, Samman et al. investigated the molecular signature of OVC and OSCC by performing low-coverage copy number (CN) sequencing on 57 OVCs and exome and RNA sequencing on a subset of these in comparison to OSCC parameters. The CN results indicated that OVCs lacked the classical OSCC patterns such as gain of 3q and loss of 3p; also, fewer genomic rearrangements were seen in OVC compared to OSCC cohort. According to exome sequencing, OVC samples lacked mutations in genes commonly associated with OSCC (TP53, NOTCH1, NOTCH2, CDKN2A and FAT1) [50]. Saumyaranjan Mallick et al. indicated that higher levels of Ki-67 were seen in SCC cases and this level was lower in VC and VH cases, respectively [49]. In 2014, Wang et al. aimed to identify differential gene expression profiles between OVC and OSCC. Gene expression analysis revealed a total of 109 altered genes in OVC compared with its matched normal oral mucosa (OVCN). Also, a total of 167 altered genes in OSCC compared with OVC were revealed by gene expression [26].

In another study, the expression levels of p53 and Ki-67 and their role in the biological behavior of SCC and VC were assessed; there was a significant difference between VC and low grade SCC regarding the expression of p53 ($p < 0.000$), but there was no such difference for Ki-67 ($P = 0.3$); there was a significant statistical difference for p53 expression between VC and high grade SCC ($P < 0.000$) but not for Ki-67 ($p = 0.9$) [32].

In 2012, Odar et al. investigated the expression of microRNA smiR-21, miR-31, miR-203, miR-125a-5p, miR-125b, phosphatase and tensin homologue (PTEN) protein and p63 in VC of the head and neck. Their results suggested higher miR-31 levels in the discrimination of VC from normal epithelium ($p < 0.001$), lower miR-125a-5p levels for discrimination of VC from WDSCC and MDSCC ($p < 0.001$) and higher miR-203 levels for differentiation of VC from PDSCC ($p < 0.001$) [47]. In 2011, Wang et al. carried out a proteomic analysis of OVC. Two-dimensional electrophoresis (2-DE) gel imaging showed that 74, 36 and 31 differential protein spots were found between OVC and OSCC, and OVC and adjacent normal oral tissue [41].

In 2010, Lin et al. assessed the expression of p53, MDM2, p21, heat shock protein 70 and HPV16/18 in OVC and oral VH. The results indicated that the mean labeling indices of p53, MDM2, p21,HSP 70, and HPV 16/18 E6 proteins in OVC samples were 21%, 31%, 7%, 17%, and 0.5%, respectively, and indices in oral VH samples were 19%, 35%, 11%, 14%, and 0.3%, respectively ($p = 0.575$, $p = 0.416$, $p = 0.053$, $p = 0.252$, $p = 0.769$, respectively) [39].

In a study by Patil et al., Cyclin B1 overexpression was compared among histological grades of OSCC, and a comparison was also made with VC. A statistically significant difference was observed among different grades of OSCC and its grades with VC [48].Terada et al. reviewed the histopathology of 10 cases of OVC and showed that immunohistochemically, p53 protein was positive in all 10 cases with its location near the basal cells and microinvasive parts. Also, Ki-67 positive cells were mainly among the basal cells and in the microinvasive areas [46]. In another study by the same group of researchers in 2011, positive p53 protein was seen in all VC cases and Ki-67 positive cells were seen mainly among the basal cells in microinvasive parts; the labeling index ranged from 12 to 21% [44]. Ray et al. demonstrated overexpression of SOD 2 and NQO1 in OVC and OSCC [35].

Zargaran et al., immunohistochemically determined the expression of Ki67 in OVC and well-differentiated OSCC. They indicated a significant difference in Ki67 expression based on the pattern of distribution of positively immunostained cells, with quantitative and semi-quantitative analyses, among four groups of epithelial hyperplasia with no dysplasia, OVC, micro invasive OSCC and well-differentiated OSCC and between epithelial hyperplasia with no dysplasia in each of the other three groups ($p = 0.0001$) [45].

Quan et al. assessed the antiapoptotic role of αB-crystalline in OVC, and immunohistochemical staining was used to evaluate expression levels. The results indicated that αB-crystalline was detected in OVC, OSCC and normal oral mucosa (NM). The expression in OVC was higher compared to that in NM ($p = 0.10$), but lower compared to OSCC, indicating that OVC was less aggressive than OSCC ($p = 0.044$) [42].

Another study investigated whether OVCs and OSCCs were characterized by differences in chromosomal instability (CIN) biomarkers. They showed that DNA aneuploid sublines were detected in more than half the OVC cases (66.7%) and in most of OSCC cases (80.0%). Multiple DNA aneuploid sublines were observed, respectively, in 2 of 6 (33.3%) DNA aneuploid OVCs and in 14 of 20 (70%) DNA aneuploid OSCCs ($p = 0.163$) [40].

Spíndula-Filho et al. investigated the cellular proliferation of SCC and VC by assessing biomarkers such as Ki-67 and Cyclin B1. A significant difference was observed in Cyclin B1 expression in the SCC group compared with VC ($p < 0.05$). However, there was no difference in Ki-67 expression between VC and SCC ($p > 0.05$). Tran et al. analyzed the hyper methylation of RASSF1A and p16INK4a by the MSP in 36 cases of oral carcinoma associated with betel chewing in Vietnamese patients and showed that hyper-methylation of p16IKN4a was detected in 63% of SCC and 67% of VC cases [37].

Adegboyega et al. conducted a study to evaluate the usefulness of five cell cycle and apoptosis-related regulatory proteins [Ki67, p16, p21, p53 and retinoblastoma gene product [RBGP)] for the diagnosis of VC in tissue sections. The results indicated overlapping in the expression of p16, p21, and RGBP in all the experimental groups (OVC, OSCC and acanthosis), being present in over half the thickness of the epithelium in 50% to 100% of cases in each study group. In one study, the expression of histone H3 mRNA and p53

protein, Cyclin D1 and B1 was assessed in VC, squamous papilloma (SP) and hyperkeratotic lesions (HK). Cyclin B1 and D1 and P53 expression levels were the highest in the SP group (85%, 78.6% and 86.4%, respectively). The H3 mRNA expression levels were the highest in the VC group (90.9%) [36].

Discussion

In this review, current articles on molecular markers in differential diagnosis of OVC were assessed. Most included studies investigated proliferative and apoptotic biomarkers namely p53 and Ki67. Due to the variability of factors and lack of significant expression of epithelial biomarkers, nothing can be concluded. However, it seems that CK10 can be useful for differentiation of OVC and benign squamous lesions. Among connective tissue biomarkers, MMP-2, −9, CD31 and CD68 seem to be useful for differentiation of OVC and OSCC, and GLUT-1 can be used for differentiating OVC from oral epithelial dysplasia.

The differential diagnosis of OVC is one of the most challenging cases among oral pathologists. Clinicohistopathological similarities between the wide spectrum of verruciform lesions like VH to noninvasive OVC and invasive well-differentiated OSCC make the diagnosis difficult. Verrucous hyperplasia and VC are similar both clinically and histopathologically [14, 51, 52]; however, invading the underlying connective tissue is one of the few differences between them [53]. Another difficulty is the insufficiency and inaccuracy of biopsy specimens, which are taken from the wrong part of the lesion; therefore, a close cooperation between clinician and pathologist is required. On the other hand, a hybrid form of VC including some foci of conventional SCC has been reported [54–56]. An accurate diagnosis is essential for a proper treatment as the common treatment of OVC is surgical excision with chemotherapy only or chemotherapy and radiotherapy [9, 57–59]; but the application of radiotherapy alone is contraindicated [9, 59]. Attempts have been made to define criteria and molecular approaches for the diagnosis of OVC, but they are not applied routinely in diagnosis and all have been limited to research. Apparently these molecular markers help to achieve an accurate and quick diagnosis.

Tumor markers are currently used for screening, detection, prediction and monitoring of the results of treatment [60] and influence the clinical decision-making [61]. Tumor markers can be defined as qualitative or quantitative changes in an existing molecule, product or mechanism. This wide range includes gene and RNA overexpression and mutation as well as overexpression of their products and mechanisms that control cellular responses or growth [62]. An appropriate tumor marker should have high specificity, sensitivity and differentiation capability between neoplastic and non-neoplastic tissues [63].

Cytoskeletal biomarkers like CK and basement membrane markers [64] are investigated in VC due to its common presentation as an exophytic superficial lesion by overexpression of keratin and lack of invasion [10, 58]. The CKs are the main fundamental elements of the cytoskeleton in the epithelium and their overexpression is frequently related to normal epithelium and their neoplasms [65–67]. Oliveira et al., [31] mainly investigated the biological behavior of OSP and OVC by assessment of CK 10, 13, 14, and 16 expression in different cell layers but Gao et al. [28] evaluated CK 20 expression in only the basal layer and demonstrated its overexpression in both OVC and OSP. Moreover, Oliveira et al. [31] reported these CKs in supra-basal to superficial layers except for CK 13 in OVC.

β-catenin is one of the epithelial to mesenchymal transition molecules that seems to play a role in metastasis [68, 69]. It seems that β-catenin as a structural molecule mainly works as an adhesive [70]. Although OSCC shows a more aggressive and metastatic pattern, there was no significant difference between OVC and OSCC inβ-catenin positivity. Laxmidevi et al. showed that β-catenin expression was only membranous in 83.3% of OVC cases while this rate was 40% in OSCC cases. Variability in pathological behavior and prognosis of these lesions can be explained with this observation [30]. In two studies, results indicated no significant differences between OVC and OSCC in type IV collagen immunohistochemical staining [33, 34]. The authors explained this contrast by differentiation between degradation enzyme secretion and migration ability that are necessary for invasion. Moreover, it seems that reduction of synthesis and the ability of regeneration [71–73] can intensify type IV collagen discontinuity.

GLUT-1 as a helper molecule, which regulates energy metabolism [74] was highly expressed in both VC and SCC, which indicates the potentially high metabolism of both lesions [25]. Previous studies showed its presence in granulation tissues [75–79] but it did not present in all VC cases [29]. Therefore, this marker is not appropriate for single application, and other markers should be sought in combination to help diagnosis. MMP-2 and -9 can also indicate metastasis and invasion [80]. The main concern is the differential diagnosis between low-grade SCC and VC; Mohtasham et al. demonstrated significant differences between low-grade SCC compared to VC in expression of MMP-9 [81]. This fact may be helpful for detection of SCC foci in conventional VC, which needs to be investigated. Ray et al., in an immunohistochemistry-based comparative study between two cases suggested MMP-9 as a potential tumor marker [35]. Density and positive area for CD68 (marked tumor-associated macrophages) and CD31 (marked microvessel density) were significantly higher in OSCC compared to OVC

[27]. These records indicated that the role of tumor-associated macrophages in metastasis and growth of OVC was similar to that in low-grade OSCC, and it seemed that increased infiltration of tumor-associated macrophages was related to pathological grades of the lesion and increased microvessel density.

In two similar studies, laminin-5 immunohistochemical staining was significantly lower in OVC compared with OSCC [33, 34]. This overexpression in OSCC might indicate that laminin-5 was overproduced by malignant cells in tumor invading front.

Various cellular changes occur during the development of neoplasms and a wide variety of these changes can be assessed by proliferative and apoptotic markers [82]. These markers mainly dysregulate cell cycle [83–85]. Cell proliferation and apoptosis are regulated and monitored by a plenty of proto-oncogenes and tumor suppressor genes such as P53, Ki67, Cyclin B1 and D1 [86].

It has already been proven that Ki-67 (MIB-1) is one of the indicators, which can act as a prognostic factor in various tumors including SCC of the oral cavity [87]. In 2014, Mallick et al. indicated a significant increase in MIB-1 staining as an endothelial proliferative index in OSCC compared to OVC [49]. However, Filho et al., and Mohtasham et al. did not mention significant differences in Ki67 expression between OSCC and OVC [32, 43]. These findings may be due to the lack of specimens. In addition, Zargaran et al., and Adegboyega et al. found similar pattern of Ki67 expression to facilitate differential diagnosis of epithelial hyperplasia from OSCC. Zargaran et al. did not find any significant diagnostic relationship between Ki67 expression and OSCC compared to OVC [38, 45]. They demonstrated that p53 expression increased progressively from normal mucosa toward OVC and reported that p53 proteins in the invasive front of OSCC were associated with the histological grade of the lesions. Presence of p53 proteins in the invasive front was also confirmed by other studies [32, 38, 46]. On the other hand, Lin et al. indicated similarity in the expression patterns of p53 between OVC and OVH. This fact may indicate an intimate association between these lesions [39].

Overexpression of Cyclin B1 seems to be related to tumor progression and histological stages [43, 48]. Nishikawa et al. showed existence of Cyclin D1 and B1 was compared in normal mucosa and the results demonstrated predominantly stained Cyclin D1 in the parabasal layer and Cyclin B1 in the basal layer [36]. This fact can be explained by the priority of expressions in the cell cycle [88]. In 2012, Odar et al. reported upregulation of miR-21, miR-203 and miR-31 among OVCs and down-regulation of them in OSCC ($p < 0.001$). In addition, previous studies had shown that elevated levels of miR-203 were related to anti-invasive, anti-proliferative and anti-metastatic behavior of tumors [89–93].

Quan et al. reported that inhibition of the activation of caspase-3 may be a part of anti-apoptosis function of αB-crystalline in OVC and these results were not statistically significant in OSCC [42]. In other studies, different approaches were considered to seek diagnostic or prognostic molecular factors in OVC. In 2015, Samman et al. published a study to assess the whole genomic architecture and transcriptomic of OVC in order to differentiate pure OVC from classical OSCC to provide a beneficial diagnostic biomarker. Finally, based on exome and RNA sequencing, they claimed that OVC and OSCC can be clearly differentiated [50]. In addition, Wang et al., in two different genomic and proteomic investigations showed 8 differentially expressed genes and 10 protein overexpression spots among OVC specimens compared to OSCC [26, 41]. Based on these studies, chromosomal instability, loss of heterozygosity at 3p and 9p and hypermethylation of p16IKN4a were not significantly different between OVC and OSCC [37, 40].

In conclusion, the review of these studies shows that although over 5 decades have passed from description of VC, its diagnosis still remains a challenge to clinicians and pathologists. While previous studies defining criteria and diagnostic approaches, especially molecular approaches, were helpful, there is still a need for a precise and reliable molecular approach.

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