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Biomarkers of progression in oral leukoplakia

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ABSTRACT

Objective: Identification of prognostic biomarker candidates for stratification and long-term surveillance of oral leukoplakia progressing to cancer via a systematic literature review.

Materials and Methods: Systematic searches with no date restrictions were conducted on March 29, 2018, targeting the databases PubMed (Ovid), EMBASE (Ovid), EBM (Ovid), and Web of Science (ISI). Bias was assessed using the Quality in Prognosis Studies tool. Biomarkers were stratified based on hallmarks of cancer.

Results: Inclusion criteria were met by 25/3415 studies. A range of biomarkers were evaluated experimentally for risk stratification, prognosis, and surveillance of oral leukoplakia in tissue, blood, and saliva. However, the studies were highly heterogeneous and require further validation. Biomarkers reported in these studies included inflammatory or oxidative markers, growth factors, ion channels, genetic and cellular regulatory factors, and epigenetic biomarkers. Studies tended to include small sample sizes, under-reported or variably reported histopathological data, did not address potential confounding, reported limited/variable follow-up data or lacked a control group. Inclusion of subsets from chemoprevention trials may have introduced bias regarding reported malignant transformation rates and accuracy of prognostic biomarkers.

Conclusions: This review identified insufficient longitudinal evidence to support validated prognostic biomarkers for oral leukoplakia. Further studies are needed to identify molecular targets with the potential to mitigate risk of malignant transformation.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) continues to have significant clinical and economic impact at the international level. It is associated with a high rate of mortality, which often is a consequence of delayed diagnosis due to lack of screening programs, low health literacy relative to OSCC, and lack of access to care. The World Health Organization (WHO) projected 354,864 new cases of oral cavity and lip cancer in 2018 (Bray *et al.*, 2018).

Oral potentially malignant disorders (OPMD), of which leukoplakia is one of several phenotypes, represent a group of conditions and lesions with variable propensity for oncogenic potential (Warnakulasuriya *et al.*, 2007). In the era of precision medicine, there is burgeoning interest in defining and characterizing relative risk for oral cancer emergence in association with OPMD. As the understanding of the molecular pathogenesis of oral cancer continues to expand, there is active interest in identifying biomarkers that could provide ability for clinicians to

longitudinally track key molecular signals associated with OPMD, and intervene prior to neoplastic transformation. The overarching aims of the systematic reviews performed by the Precision Medicine Group of World Workshop on Oral Medicine VII were to:

- Assess if prognostic biomarkers could accurately stratify the risk of malignant transformation of oral leukoplakia.

- Assess the relationship between prognostic biomarkers and the patient's risk profile including lesion clinico-pathologic characteristics in addition to patient's risk factors.

- Evaluate whether biomarkers could independently predict malignant transformation of oral leukoplakia.

- Establish the minimum follow-up intervals required for biomarkers to predict malignant transformation.

- Assess the efficacy of the investigated biomarkers and management protocol.
  Formulate an algorithm that would help clinicians to provide the best supported evidence-based management protocol to patients with oral leukoplakia.

After oral submucous fibrosis, oral leukoplakia is the most common OPMD, with a worldwide prevalence of 4.11% (95% CI: 1.98-6.97) (Mello *et al.*, 2018). Leukoplakia has been defined by the WHO as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" (Warnakulasuriya *et al.*, 2007). Risk factors for oral leukoplakia are similar to those observed in oral cancer and include tobacco smoking, heavy alcohol consumption, areca nut chewing (especially in South Asian countries), immunosuppression (e.g., HIV/AIDS, post-organ transplantation), personal or family history of cancer (60-70%), ultraviolet light exposure (for lip lesions only) and selected syndromes (e.g., dyskeratosis congenita) (Villa & Woo, 2017), (Warnakulasuriya, 2018).

Leukoplakia is a clinical diagnosis, most commonly presenting in two main phenotypes: *homogeneous* and *non-homogeneous* leukoplakia. *Proliferative verrucous leukoplakia* represents a third, rarer, high risk subtype (Warnakulasuriya, 2018). Irrespective of type of oral leukoplakia, the gold standard for final diagnosis remains incisional biopsy. Risk of malignant transformation depends on the clinical form and the grade of dysplasia, although other clinical and histopathological parameters have been reported as drivers (Speight *et al.*, 2018). Non-

homogeneous leukoplakias carry a 20%–25% risk of cancer progression *versus* 0.6% – 5% in homogeneous cases (Napier & Speight, 2008, van der Waal & Axell, 2002, Reibel, 2003).

A key step to better understanding oral leukoplakia outcomes is to identify the molecular factors that drive malignant progression, as these factors may also represent attractive candidates for targeted therapies. With the advent of precision medicine, a growing evidence base has explored predictive and prognostic biomarkers for oral leukoplakia. This paper systematically reviewed longitudinal studies which specifically aimed to: 1) assess whether prognostic biomarkers could accurately stratify the risk of progression of oral leukoplakia to cancer, and 2) evaluate the reliability of biomarkers in long-term surveillance of oral leukoplakia. Future studies will focus on the other overarching aims as mentioned above.

MATERIALS AND METHODS

This study was conducted by the Precision Medicine Work Group within the World Workshop on Oral Medicine VII (WWOM VII). Results are reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher *et al.*, 2009b). PICO (**P**atients, **I**ntervention/exposure/prognostic factor, **C**omparison group and **O**utcome) was used to formulate the research question.

- **P**atients: patients with oral leukoplakia

- **I**ntervention/Exposure/Prognostic factor: Biomarkers in human specimens (saliva, blood, gingival crevicular fluid, oral tissues)

- **C**omparison: healthy controls or patients with oral squamous cell carcinoma

- **O**utcome: squamous cell carcinoma

- **St**udies: longitudinal (prospective) studies

Study selection

Studies that were potentially eligible for inclusion evaluated biomarker expression in: (i) human specimens (saliva, blood, gingival crevicular fluid, oral tissues) with follow-up data (over time); (ii) patients with oral leukoplakia compared to healthy controls; or (iii) patients with oral

leukoplakia compared to patients with oral squamous cell carcinoma.

Studies were excluded if they were: 1) studies investigating only non-human tissues, 2) withdrawn/retracted studies, 3) reviews, 4) case reports, 5) commentaries, 6) opinion articles, 7) letters to the Editor, or 8) congress abstracts.

The selection of studies for review was conducted in the following five steps as illustrated in **Figure 1**.

Step 1: Electronic literature searches were conducted on March 29, 2018, using the PubMed (Ovid), Embase (Ovid), Evidence Based Medicine (EBM) Reviews (Ovid), and Web of Science (ISI) databases with no publication year restrictions. The search strategies according to the syntax rules of each database are displayed in **Supplementary Table S1**. The identified citations were imported into the electronic database Endnote X8 (Clarivate Analytics, Philadelphia, PA, USA). De-duplication was achieved by the Endnote software and manually by the two reviewers (AVil, AC).

Each subsequent step was conducted by two blinded reviewers (AVil, AC) to exclude records ineligible for inclusion, based on sequential review of title only, title and abstract, and finally full text (Step 4). The applied exclusion categories are shown in **Table 1.** When more than one exclusion category would pertain to a study, the most important was selected. At each step, review discordance between the reviewers was resolved by discussion between the two reviewers (AVil, AC) under supervision of the senior reviewer (CSF).

Step 2: Among unique records screened by title, only 1,006 out of 3,145 were retained. Cohen's kappa statistic for inter-reviewer agreement was 0.95 (95% CI: 0.94-0.96) and the absolute agreement between the two reviewers was 96.7%.

Step 3: Screening by both title and abstract resulted in retention of 749 of 1,006 for full text review. The absolute agreement between the two reviewers was 96.7% and the kappa statistic was 0.92 (95% CI: 0.91-0.94).

Step 4: Based on review of the full text, 331 of 749 were retained based on identification of "leukoplakia" as the definitive diagnosis. The reasons for excluding the 418 studies are shown in **Table 1**.

Step 5: The remaining 331 papers were allocated to one or more of the following eligibility categories, which respectively assessed the efficacy of biomarkers in:

- Y1: stratifying the risk of progression of oral leukoplakia to cancer (prospective longitudinal studies only).

- Y2: long-term surveillance of oral leukoplakia (longitudinal studies only).

- Y3: diagnosis of oral leukoplakia as an adjunct to oral examination (prospective case-control/cross-sectional studies only)

- Y4: progression of oral leukoplakia in a retrospective data set (case-control/cross-sectional studies only)

- Y5: differentiating oral leukoplakia from controls; correlation of differential biomarker(s) expression with diverse clinico-pathological parameters (case-control/cross-sectional studies only).

Inter-reviewer absolute agreement for Step 5 was 85.47% and the kappa statistic was 0.59 (95% CI: 0.50-0.68), but 100% absolute agreement was reached upon a second revision.

As per title, the current report was limited to the 25 longitudinal studies included in the categories Y1 regarding progression risk and Y2 on long-term surveillance. Consequently, 306 of 331 studies classified by Y categories that did not meet eligibility for inclusion in Y1 or Y2 categories due to either lack of eligibility for any of the Y category or because they were included into categories Y3 – Y5, were excluded from further review in the current report. Of note, all papers included in the Y2 category were also included in the Y1 category and the results will therefore be reported collectively.

The reviewer (AVil) and the consultant (CSF) extracted and entered into specifically developed forms (Microsoft Excel 2010, Redmond, Washington, USA) all relevant data from the selected papers. Risk of bias for the 25 included studies was assessed independently by one reviewer (AVil) and the consultant (CSF) using the '**Qu**ality **i**n **P**rognosis **S**tudies' (QUIPS) tool (Hayden *et al.*, 2013), which evaluates the six domains 'study participation', 'study attrition', 'prognostic factor measurement', 'outcome measurement', 'study confounding', and 'statistical analysis and reporting'. Discord was resolved by consensus.

Statistical analysis

Absolute percent inter-reviewer agreement and Cohen's kappa coefficient were calculated using IBM Statistics 23 (SPSS, Chicago, Illinois, USA). The heterogeneity of the studies and the high number of different biomarkers studied prevented any quantitative analysis of the results, so no meta-analysis was performed.

RESULTS

Of the 331 publications eligible for the categories Y1 – Y2 out of the originally identified 3,415 unique records, 25 studies were allocated to groups Y1 and Y2 and therefore included in this report (**Figure 1)**. Results are reported by types of biomarkers stratified to the hallmarks of cancer (Hanahan & Weinberg, 2011). The main characteristics of the studies are presented in **Table 2,** and a detailed description of the biomarkers identified is reported in the **Supplementary Results.**

Types of biomarkers

Biomarkers reported in these studies (**Table 2**) included inflammatory or oxidative markers (Chang et al., 2013, Massarelli et al., 2005b, Rai *et al.*, 2010), growth factors (Beenken *et al.* 1994, Beenken *et al.*, 1999, Wan *et al.,* 1999, Uehara *et al.,* 2010) cell signaling biomarkers (Saintigny *et al*., 2011, Saintigny *et al*., 2018, Saintigny *et al*., 2018, Sakata *et al*., 2017) genetic and cellular regulatory factors (Lee *et al*., 2000, Nagao *et al*., 2017, Tanić *et al.,* 2009, Visioli *et al*., 2012, Mao *et al*., 1996), ion channels (Fernandez-Valle *et al.*, 2016a, Fernandez-Valle *et al.*, 2016b) sustained angiogenesis factors (Nayak *et al.*, 2015, Yang CZ *et al.*, 2014, Kawaguchi *et al*., 2008, Saintigny *et al.*, 2009, Lin *et al*., 2010,) and epigenetic biomarkers (Xiao *et al*., 2012, Yang *et al*., 2013, Foy *et al*., 2015). Studies tended to include small sample sizes, under-reported or variably reported histopathological data, did not address potential confounding, reported limited/variable follow-up data or lacked a control group. Notably, 14 of the 25 studies were components of chemoprevention trials (Saintigny *et al.*, 2009, Mao *et al*., 1996, Saintigny *et al.*, 2011, Uehara *et al*., 2010, Massarelli *et al.*, 2005b, Saintigny *et al.*, 2018, Foy *et al*., 2015, Nagao *et al.*, 2017, Kawaguchi *et al.*, 2008, Rai *et al.*, 2010, Lee *et al.*, 2000, Yang *et al.*, 2013, Wan *et al*., 1999, Beenken *et al.*, 1994). Inclusion of subsets from chemoprevention trials may have

introduced bias regarding reported malignant transformation rates and accuracy of prognostic biomarkers.

Quality assessment of the studies

Low risk of bias was observed in 'statistical reporting', 'study participation', 'study attrition,' and 'outcome measurement' (81%, 70%, 52% and 52% of included studies, respectively); while moderate risk of bias in 'study confounding' was found in 70% of studies and in 'outcome measurement', 'prognostic factor measurement,' and 'study attrition' in 48% of the studies. Percentage of studies with high risk of bias was relatively low and varied between 0 and 11% across the different domains **(Figure 2; Table 3).**

DISCUSSION

This systematic review was directed to identification of biomarkers that could predict the likelihood of malignant progression over time in patients affected by oral leukoplakia (**Table 4**). Therefore, only studies applying longitudinal designs were included. Overall, the 25 included studies documented a wide range of biomarkers derived from three different anatomic sources: serum, tissue, and saliva. Heterogeneity of biomarkers investigated across these studies precluded direct inter-study comparison in virtually all cases.

Whereas selected biomarkers that were examined longitudinally seem promising, major limitations delineated in the current studies prevent definitive clinical application at this time. Most of the studies: 1) included small sample sizes with the largest comprising only 162 patients (Kawaguchi *et al.*, 2008); 2) suffered from a paucity of histopathological data for leukoplakia and a lack of reporting of demographics and potentially confounding factors such as age, tobacco smoking, and alcohol consumption **(Table 2)**; 3) reported on limited and variable follow-up data and/or did not include a control group. As such, no negative or positive predictive value was reported for the biomarkers. Each study included in the review evaluated distinct biomarkers, with the exception of podoplanin that was evaluated by two research groups

(Saintigny *et al.*, 2009, Kawaguchi *et al.*, 2008), but continues to require further investigation. Thus, potential validity of biomarkers examined to date remains to be demonstrated in other studies. Several included papers reported on biomarkers examined in the context of chemoprevention trials (14/25 studies) where intervention may have potentially impacted the malignant transformation rates and accuracy of prognostic biomarkers relative to risk detection for malignant progression of oral leukoplakia (Saintigny *et al*., 2009, Mao *et al*., 1996, Saintigny *et al.,* 2011, Uehara *et al.,* 2010, Massarelli *et al*., 2005, Saintigny *et al.,* 2018, Foy *et al.,* 2015, Nagao *et al.,* 2017, Kawaguchi *et al*., 2008, Rai *et al*., 2010, Lee *et al.,* 2000, Yang *et al.,* 2013, Wan *et al*., 1999, Beenken *et al*., 1994). Finally, most studies exhibited a moderate risk for bias in association with missing data, no consideration of potential confounding, and availability of limited follow up data **(Table 3)**. Future studies should include a longer follow-up (the longest one reported in the studies included for this systematic review was 7.5 years; by Saintigny *et al*., 2009) to capture all cases of oral leukoplakia that undergo malignant transformation. This requires a larger effort with multiple centers and resources involved. Notably, biomarker assessment was not conducted in a blinded manner in any of the included studies, further introducing additional potential for bias.

Ten studies aimed to identify objectively measured molecular biomarkers for potential utility in identifying patients with oral leukoplakia at higher risk of malignant transformation. The remaining 14 studies that incorporated biomarker assessment as part of chemoprevention trials, had other objectives. We defined the potential role of each of the biomarkers reported in these studies based on their relational juxtaposition to essential hallmarks of cancer (Hanahan & Weinberg, 2011).

Based on evidence available to date, candidate biomarkers for cancer progression in patients affected by leukoplakia examined in this systematic review lacked substantive evidence as harbingers of risk for malignant transformation of oral leukoplakia. However, we only included those studies that specifically described "leukoplakia"; studies that described dysplasia only were excluded. Consequently, some biomarkers with prognostic value for oral dysplastic lesions (and possibly some leukoplakia cases) may have been missed. In addition, some of these biomarkers were captured in the Y3, Y4 or Y5 categories and will be reviewed separately in future publications by our group. One example of such a biomarker comes from the study by Zhang and colleagues who examined 296 oral premalignant lesions and showed that LOH at 3p and/or 9p was present in 20% of cases that underwent malignant transformation (Zhang *et al.*,

2012), further supporting the role of LOH in malignant transformation of oral leukoplakia. Although LOH may indeed be a predictive biomarker for malignant transformation, this systematic review highlights that both clinical and histopathological descriptors of lesions should be included in future studies on the topic to ensure robustness of data, but also to allow subsequent meta-analyses should more than one study investigate the same marker or set of markers.

This systematic review disclosed that insufficient longitudinal evidence is currently available to support identification of biomarkers that could improve current methods for detection of leukoplakia and any subsequent malignant disease progression. Therefore, the strength of the current evidence precludes advancement of any candidate biomarkers examined to date into clinical practice. While a variety of technologies and laboratory techniques were employed to measure biomarker expression (including IHC, PCR, ELISA, and next-generation sequencing), outcomes of these studies based on the current body of evidence does not achieve the standards required to identify and validate candidate biomarkers investigated to date. While recent advancements in biomarker development using microarray technology, mass spectrometry, next-generation sequencing and proteomic technologies have facilitated accomplishment of relatively rapid screening in real time, one of the major challenges that remains unaddressed is the validation in large longitudinal trials to assess the true effectiveness of these biomarkers. Reliable, sensitive and specific biomarkers are needed to predict malignant transformation in patients affected by leukoplakia and inform physicians on correct treatment decisions. Thus, their relative value remains equivocal pending validation in future appropriately designed studies, with no advances currently observed. Well-designed randomized controlled studies with proper follow-up are required to propose and validate biomarkers for the purpose of establishing a new basis for their clinical and diagnostic utility. Consequently, clinicians continue to rely on tissue biopsy and histopathological assessment of oral dysplasia as the gold standard to determine the risk of malignant transformation and management strategies for patients with oral leukoplakia.

Limitations in tissue availability of leukoplakia samples (often small, formalin-fixed paraffin embedded) and the pre-invasive nature of leukoplakia may limit its systemic detectable expression in saliva and blood. Two promising biomarkers identified in this systematic review included podoplanin and cell cycle regulators. Podoplanin was identified as a possible

independent predictor for cancer progression (hazard ratio = 3.1; 95% CI: 1.5-6.2) (Kawaguchi et al., 2008) and p27 loss was shown to be an independent factor for malignant transformation (p=0.02) (Massarelli et al., 2005a). However, despite the potential for greatly improving current clinical tools and collective recent advances, there is still insufficient longitudinal evidence to support the identification of these and other biomarkers that could improve the current methods for detection of leukoplakia and any subsequent malignant disease progression based on the 25 studies that met eligibility for inclusion in this systematic review. Thus, oral epithelial dysplasia on histopathological assessment of a biopsy specimen currently remains the best predictor for transformation to invasive squamous cell carcinoma.

Future directions

The past decade has seen an increase in availability and access to high-throughput profiling and sequencing technologies for genomic analysis and increased bioinformatic approaches to support rapid and comprehensive investigations of multi-omics data in the pre-cancer and cancer research domain. The growing capacity for integration and systems analysis of multi-omics data needs to be pursued to help elucidate interactions between genetic and epigenetic alterations being recognized in leukoplakia and promote identification of a prognostic biomarker or panel of biomarkers with improved potential for cancer prediction and simultaneously, definition of pathways involved in malignant transformation including candidate molecules which could be targeted by therapeutic interventions to impede progression of oral leukoplakia to invasive cancer. Notably, the era of precision medicine has seen achievement of this goal in the context of some cancers, including breast cancer. Achievement of this goal is further linked to definition of specific genetic alterations associated with leukoplakia and processes that drive malignant transformation. Increased understanding of these processes will also promote further identification of candidate biomarkers that may represent detectable molecular signals arising as a consequence of transformational processes. In the future, additional well-designed research that defines triggers of leukoplakia progression through integrated, *ad-hoc* computational analyses are needed to leverage genomic and other meta-data in defining appropriate candidate biomarkers and tailored molecular targets with translational potential to mitigate risk of malignant transformation associated with leukoplakia.

As more high-throughput technologies such as next-generation sequencing are utilized in the discovery of diagnostic, prognostic and predictive biomarkers, it is incumbent on researchers to better understand biomarker types and to better plan study designs to suit the biomarker type but also the question at hand. In the case of oral leukoplakia malignant transformation, assessing DNA tissue biomarkers through genome-wide association studies or even exome sequencing in affected individuals may provide insight into genetic mutations or aberrations of susceptibility in affected individuals, and if followed-up over time, may lead to discovery of a susceptibility profile for malignant transformation, as it is known that not all leukoplakia progress to malignancy. These biomarkers can be tested retrospectively on bio-banked samples, and would hasten biomarker discovery as patient recruitment for biomarker study design would not necessarily need to proceed prospectively. DNA tumor biomarkers of oral leukoplakia samples themselves may offer equally interesting insights, but would require a prospective study design. These biomarkers would be amenable for testing therapy response and pharmacodynamics, but may suffer from lack of reproducibility as different somatic mutations may be present in different parts of the lesion, thus highlighting the importance of biopsy site selection and multiple sampling of oral leukoplakia. Nonetheless, having this insight would dramatically increase our ability to discuss treatment options with patients, and would be invaluable prognostic predictors for therapy. Finally RNA, protein or metabolite biomarkers can be assayed as DNA tumor biomarkers can, but have the advantage of being tested in a variety of bio-samples including blood, saliva, gingival crevicular fluid, tissue, or cell scrapes, would equally be amenable to therapy testing and monitoring of response, would provide reproducibility at any one point in time, could be used in prospective study designs, but additionally would be suitable as surrogate markers of disease. Furthermore, their discovery can elucidate molecular pathways involved in malignant transformation and provide more comprehensive insights into this process.

Ideally, collaborative research, at the international level, strategically designed to systematically collect and analyze data is required in order to (i) achieve a robust, adequately-powered study population for follow-up of patients with leukoplakia and (ii) observe progression in a larger subset of patients than is normally possible in single-center studies due to the relatively low rate of malignant transformation. Patients with newly diagnosed leukoplakia should be included with subsequent longitudinal follow-up, and collection of structured data at specifically-defined time-points. Future studies should also focus on

identifying biomarkers by integrating -omics data with environmental and lifestyle variables with the final goal of identifying more informative predictors of cancer progression that supersede dependence on histopathological diagnosis alone. Collection of these data into a centralized repository in the context of good histopathology data would further create a resource amenable to application of bioinformatics approaches and predictive modeling to identify the most informative variables for screening across time.

CONCLUSIONS

This review identified insufficient longitudinal evidence to support validated prognostic biomarkers for oral leukoplakia. Further studies are needed to identify molecular targets with the potential to mitigate risk of malignant transformation of oral leukoplakia to advance precision medicine approaches to management of patients with these lesions.

REFERENCES

Beenken, S. W., Huang, P., Sellers, M., Peters, G., Listinsky, C., Stockard, C., . . . Grizzle, W. (1994). Retinoid modulation of biomarkers in oral leukoplakia/dysplasia. J*ournal of Cellular Biochemistry. Supplement,* 19, 270-277. doi: N/A.

Beenken, S. W., Sellers, M. T., Huang, P., Peters, G., Krontiras, H., Dixon, P., . . . Grizzle, W. E. (1999). Transforming growth factor alpha (tgf-alpha) expression in dysplastic oral leukoplakia: modulation by 13-cis retinoic acid. *Head & Neck,* 21, 566-573. doi: 10.1002/(SICI)1097-0347(199909)21:6<566::AID-HED11>3.0.CO;2-H.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a Cancer Journal for Clinicians*, 68, 394-424. doi: 10.3322/caac.21492.

Chang, P. Y., Kuo, Y. B., Wu, T. L., Liao, C. T., Sun, Y. C., Yen, T. C., & Chan, E. C. (2013). Association and prognostic value of serum inflammation markers in patients with leukoplakia and oral cavity cancer. *Clinical Chemistry and Laboratory Medicine*, 51, 1291-1300. doi: 10.1515/cclm-2012-0504.

D'Amico, M., Gasparoli, L., & Arcangeli, A. (2013).Potassium channels: novel emerging biomarkers and targets for therapy in cancer. *Recent Patents on Anti-cancer Drug Discovery* 8(1): 53-65. doi: 10.2174/15748928130106

Fernandez-Valle, A., Rodrigo, J. P., Garcia-Pedrero, J. M., Rodriguez-Santamarta, T., Allonca, E., Lequerica-Fernandez, P., & de Vicente, J. C. (2016). Expression of the voltage-gated potassium channel kv3.4 in oral leucoplakias and oral squamous cell carcinomas. *Histopathology,* 69, 91-98. doi: 10.1111/his.12917.

Fernandez-Valle, A., Rodrigo, J. P., Rodriguez-Santamarta, T., Villaronga, M. A., Alvarez-Teijeiro, S., Garcia-Pedrero, J. M., . . . de Vicente, J. C. (2016). HERG1 potassium channel expression in potentially malignant disorders of the oral mucosa and prognostic relevance in oral squamous cell carcinoma. *Head & Neck,* 38, 1672-1678. doi: 10.1002/hed.24493.

Foy, J. P., Pickering, C. R., Papadimitrakopoulou, V. A., Jelinek, J., Lin, S. H., William, W. N., Jr., . . . Saintigny, P. (2015). New DNA methylation markers and global DNA hypomethylation are associated with oral cancer development. *Cancer Prevention Research,* 8, 1027-1035. doi: 10.1158/1940-6207.CAPR-14-0179.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell* 144(5): 646-674. doi: 10.1016/j.cell.2011.02.013

Hayden, J. A., van der Windt, D. A., Cartwright, J. L., Cote, P., & Bombardier, C. (2013). Assessing bias in studies of prognostic factors. *Annals of Internal Medicine,* 158, 280-286. doi: 10.7326/0003-4819-158-4-201302190-00009.

Kawaguchi, H., El-Naggar, A. K., Papadimitrakopoulou, V., Ren, H., Fan, Y. H., Feng, L., . . . Mao, L. (2008). Podoplanin: A novel marker for oral cancer risk in patients with oral premalignancy. *Journal of Clinical Oncology,* 26, 354-360. doi: 10.1200/JCO.2007.13.4072.

Lee, J. J., Hong, W. K., Hittelman, W. N., Mao, L., Lotan, R., Shin, D. M., . . . Lippman, S. M. (2000). Predicting cancer development in oral leukoplakia: ten years of translational research. *Clinical Cancer Research,* 6, 1702-1710. doi: N/A.

Lin, C. Y., Chen, W. H., Liao, C. T., Chen, I. H., Chiu, C. C., Wang, H. M., . . . Cheng, A. J. (2010). Positive association of glucose-regulated protein 78 during oral cancer progression and

the prognostic value in oral precancerous lesions. *Head & Neck,* 32, 1028-1039. doi: 10.1002/hed.21287.

Mao, L., Lee, J. S., Fan, Y. H., Ro, J. Y., Batsakis, J. G., Lippman, S., . . . Hong, W. K. (1996). Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. 2, 682-685. doi: n/a.

Massarelli, E., Brown, E., Tran, N. K., Liu, D. D., Izzo, J. G., Lee, J. J., . . . Papadimitrakopoulou, V. A. (2005). Loss of E-cadherin and p27 expression is associated with head and neck squamous tumorigenesis. *Cancer,* 103, 952-959. doi: 10.1002/cncr.20879.

Mello, F. W., Miguel, A. F. P., Dutra, K. L., Porporatti, A. L., Warnakulasuriya, S., Guerra, E. N. S., & Rivero, E. R. C. (2018). Prevalence of oral potentially malignant disorders: a systematic review and meta-analysis. *Journal of Oral Pathology & Medicine.* 47, 633-640. doi: 10.1111/jop.12726.

Moher, D., A. Liberati, J. Tetzlaff, D. G. Altman, The PRISMA Group (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 6(7): e1000097. doi: 10.1371/journal.pmed.1000097.

Nagao, T., Warnakulasuriya, S., Sakuma, H., Miyabe, S., Hasegawa, S., Machida, J., . . . Hashimoto, S. (2017). P53 and ki67 as biomarkers in determining response to chemoprevention for oral leukoplakia. *Journal of Oral Pathology & Medicine,* 46, 346-352. doi: 10.1111/jop.12498.

Napier, S. S., & Speight, P. M. (2008). Natural history of potentially malignant oral lesions and conditions: an overview of the literature. *Journal of Oral Pathology & Medicine.* 37, 1-10. doi: 10.1111/j.1600-0714.2007.00579.x

Nayak, S., Goel, M. M., Makker, A., Bhatia, V., Chandra, S., Kumar, S., & Agarwal, S. P. (2015). Fibroblast growth factor (FGF-2) and its receptors FGFR-2 and FGFR-3 may be putative biomarkers of malignant transformation of potentially malignant oral lesions into oral squamous cell carcinoma. *PLoS One,* 10. doi: 10.1371/journal.pone.0138801.

Rai, B., Kaur, J., Jacobs, R., & Singh, J. (2010). Possible action mechanism for curcumin in pre-cancerous lesions based on serum and salivary markers of oxidative stress. *Journal of Oral Science,* 52, 251-256. doi: 10.2334/josnusd.52.251.

Reibel, J. (2003). Prognosis of oral pre-malignant lesions: Significance of clinical, histopathological, and molecular biological characteristics. *Critical Reviews in Oral Biology and Medicine*, 14, 47-62. doi: 10.1177/154411130301400105.

Saintigny, P., El-Naggar, A. K., Papadimitrakopoulou, V., Ren, H., Fan, Y. H., Feng, L., . . . Mao, L. (2009). Deltanp63 overexpression, alone and in combination with other biomarkers, predicts the development of oral cancer in patients with leukoplakia. *Clinical Cancer Research,* 15, 6284-6291. doi: 10.1158/1078-0432.CCR-09-0498.

Saintigny, P., William Jr., W. N., Foy, J. P., Papadimitrakopoulou, V., Lang, W., Zhang, L., . . . Lippman, S. M. (2018). Met receptor tyrosine kinase and chemoprevention of oral cancer. *Journal of the National Cancer Institute,* 110, 01. doi: 10.1093/jnci/djx186.

Saintigny, P., Zhang, L., Fan, Y. H., El-Naggar, A. K., Papadimitrakopoulou, V. A., Feng, L., . . . Mao, L. (2011). Gene expression profiling predicts the development of oral cancer. *Cancer Prevention Research,* 4, 218-229. doi: 10.1158/1940-6207.CAPR-10-0155.

Sakata, J., Yoshida, R., Matsuoka, Y., Nagata, M., Hirosue, A., Kawahara, K., . . . Nakayama, H. (2017). Predictive value of the combination of SMAD4 expression and lymphocyte infiltration in malignant transformation of oral leukoplakia. *Cancer Medicine,* 6, 730-738. doi: 10.1002/cam4.1005.

Speight, P. M., Khurram, S. A., & Kujan, O. (2018). Oral potentially malignant disorders: Risk of progression to malignancy. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology,* 125, 612-627. doi: 10.1016/j.oooo.2017.12.011.

Tanic, N., Tanic, N., Milasin, J., Vukadinovic, M., & Dimitrijevic, B. (2009). Genomic instability and tumor-specific DNA alterations in oral leukoplakias. *European Journal of Oral Sciences,* 117, 231-237. doi: 10.1111/j.1600-0722.2009.00624.x.

Uehara, M., Ikeda, H., Nonaka, M., & Asahina, I. (2010). Histopathological change of oral malignant tumour and epithelial dysplasia subjected to photodynamic therapy. *Journal of Oral & Maxillofacial Research,* 1, e5. doi: 10.5037/jomr.2010.1305.

van der Waal, I., & Axell, T. (2002). Oral leukoplakia: A proposal for uniform reporting. *Oral Oncology,* 38, 521-526. doi: 10.1016/S1368-8375(01)00125-7

Villa, A., & Woo, S. B. (2017). Leukoplakia - a diagnostic and management algorithm. *Journal of Oral and Maxillofacial Surgery,* 75, 723-734. doi: 10.1016/j.joms.2016.10.012.

Visioli, F., Lauxen, I. S., Sant'ana Filho, M., & Rados, P. V. (2012). Expression of the cell cycle regulation proteins p53 and p21WAF1 in different types of non-dysplastic leukoplakias. *Journal of Applied Oral Science,* 20, 369-375. doi: 10.1590/S1678-77572012000300013

Wan, X. S., Meyskens Jr, F. L., Armstrong, W. B., Taylor, T. H., & Kennedy, A. R. (1999). Relationship between protease activity and neu oncogene expression in patients with oral leukoplakia treated with the Bowman Birk inhibitor. *Cancer Epidemiology, Biomarkers & Prevention,* 8, 601-608. doi: N/A.

Warnakulasuriya, S. (2018). Clinical features and presentation of oral potentially malignant disorders. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology,*125, 582-590. doi: 10.1016/j.oooo.2018.03.011.

Warnakulasuriya, S., Johnson, N. W., & van der Waal, I. (2007). Nomenclature and classification of potentially malignant disorders of the oral mucosa. *Journal of Oral Pathology & Medicine,* 36, 575-580. doi: 10.1111/j.1600-0714.2007.00582.x.

Xiao, W., Bao, Z. X., Zhang, C. Y., Zhang, X. Y., Shi, L. J., Zhou, Z. T., & Jiang, W. W. (2012). Upregulation of miR-31 is negatively associated with recurrent/newly formed oral leukoplakia. *PLoS One,* 7. doi: 10.1371/journal.pone.0038648.

Yang, C. Z., Ma, J., Luo, Q. Q., Neskey, D. M., Zhu, D. W., Liu, Y., . . . Zhong, L. P. (2014). Elevated level of serum growth differentiation factor 15 is associated with oral leukoplakia and oral squamous cell carcinoma. *Journal of Oral Pathology & Medicine,* 43, 28-34. doi: 10.1111/jop.12091.

Yang, Y., Li, Y. X., Yang, X., Jiang, L., Zhou, Z. J., & Zhu, Y. Q. (2013). Progress risk assessment of oral premalignant lesions with saliva miRNA analysis. *BMC Cancer,* 13, 129. doi: 10.1186/1471-2407-13-129.

Zhang, L., Poh, C.F., Williams, M., Laronde, D.M., Berean, K., Gardner, P.J., …Rosin, M.P. (2012) Loss of Heterozygosity (LOH) Profiles – Validated Risk Predictors for Progression to Oral Cancer *Cancer Prev Res (PhilA),* 5, 1081-9. doi: 10.1158/1940-6207.CAPR-12-0173.

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TABLES

Table 1. Reason for exclusion after full text assessment.

Exclusion Category	Reason for Exclusion	Number of Studies
N0	Not English language	17
N1	Not original study (review, guideline, editorial, conference abstract)	2
N2	No human tissue or only immortalized human cell lines *in vitro*	3
N3	No clinical diagnosis of oral leukoplakia, only dysplasia	332
N4	Cancer patients only: resection margins/perilesional tissue in oral/oropharynx squamous cell carcinoma; history of cancer prior to or during study; undergoing chemo or radiation therapy	6
N5	Predatory journals	2
N6	Retracted articles, or title includes "expression of concern"	3
N7	Other reason for not meeting inclusion criteria	53
All (N1-N7)	Reports ineligible for inclusion	418

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Author Year Country Study Design Duration	Bio-marker*	Specimen Type	Leukoplakia (n)	Risk Factors: Leukoplakia No. of cases /smoking or alcohol use	Age, (yr) Mean (±SD)** (Range)	Sex #M/#F	Histopathology No. of cases/type	Site of lesion: No. of cases/location	Outcomes/Results Biomarker~Malignancy Link

Table 2. Findings from the 25 eligible studies using biomarkers with leukoplakia specimens.

Tissue invasion, metastasis and inflammatory markers									
Chang et al. 2013 China Follow up: 28 months (only available for OSCC patients)	**IL-6** **M-CSF** **TGF-β1** **ICAM-1** **E-selectin** **CRP** **SAA** **MMP-2** **MMP-9**	serum	46	NA	Leukoplaki a Mean 46.7 Range: (26–72) Healthy: Mean 48.3 Range: (27-82)	44/2	NA	Leukoplakia samples: 4 tongue 30 buccal mucosa 2 hard palate 2 retromolar trigone 5 tongue+buccal mucosa 2 lip+buccal mucosa	Significant difference: TGF- β1 (AUC: 0.756, p<0.001); CRP (AUC: 0.704, p<0.001) MMP-9; (AUC: 0.729, p<0.001) in leukoplakia vs. healthy controls. Sensitivity to detect leukoplakia or OSCC following evaluation of MMP-9, MMP-2, TGF-β1, CRP, and E-selectin: 67.4% and 90%, respectively.
Massarelli et al. 2005 USA Follow up: median of	**p27 &** **E-cadherin** **protein** **expression**	tissue (biopsy)	31	Smokers: E-cadherin leukoplakia group (laryngeal/or al): 26 former 11 current	E- cadherin group: Mean 58.3 p27 group:	E-cadherin group: 15/31 p27 group: 14/26	E-cadherin group: 5 hyperplasia/ hyperkeratosis Dysplasia: 13 mild 16 moderate 12 severe	NA	21 mo post follow up: Loss of E-cadherin expression 25/46 (54%) Development of OSCC: 14/23 (61%), No. who developed OSCC with:

28 months			20 never p27 leukoplakia group (laryngeal/oral) 23 former 8 current 9 never	mean 57.3		p27 group: 5 hyperplasia/ hyperkeratosis Dysplasia 13 mild 12 moderate 10 severe		- leukoplakia (14/31 (45%)). - p27 loss (13/19 (68%)) - p27 retention: (7 /21 (33%)). p27 loss was an independent predictor of malignant transformation (log-rank test; P=0.02). E-cadherin and p27 loss: was associated with a decreased time to disease progression (log-rank test; P=0.04).
Serum oxidative markers							

Rai et al. 2010 India Follow up: 181 days	**MDA** **8-OHdG** **vitamin C** **vitamin E**	saliva serum	25	NA	Age range: 17-50	13/12	NA	NA	Compared with healthy controls, - MDA and 8-OHdG levels were increased in oral leukoplakia, submucosal fibrosis and oral lichen planus cases, (p<0.05); serum and salivary vitamin C and E levels were decreased (p<0.05). Mean salivary and serum MDA, 8-OHdG, and vitamin C and E levels were significantly different in patients with oral leukoplakia, submucosal fibrosis and oral lichen planus before the intake of curcumin and after

								treatment (p<0.05).

Proliferation without exogenous stimulation

Growth factors

Beenken et al. 1994 USA Follow up: 3 months	**EGFR** **TGF-α** **HER-2/neu**	tissue (biopsy)	9	NA	NA	NA/NA	Dysplasia: 5 mild 1 moderate 3 severe	NA	Pretreatment expression scores for EGFR, TGF-a, and HER-2/neu in leukoplakia were increased when compared to their expression scores in normal-appearing mucosa. TGF-α expression ratios in leukoplakia were significantly reduced after treatment with 13-cis-retinoic acid.

Beenken et al. 1999 USA Follow up: 1 month	**TGF-α** **EGFR**	tissue (biopsy)	11	NA	NA	NA/NA	Dysplasia: 5 mild 3 moderate 3 severe	NA	TGF- α and EGFR scores were increased in dysplastic oral leukoplakia specimens when compared with adjacent normal appearing mucosa. TGF-α expression scores in normal-appearing mucosa adjacent to dysplastic tissue were higher than those seen in healthy controls.
Wan et al. 1999 USA Follow up: 1 month	**neu proteins**	blood & oral mucosa cells	25	NA	NA	NA/NA	NA	NA	Changes in the protease activity in oral mucosal cells after therapy correlated with the changes in the neu protein levels in oral mucosal cells (p< 0.001) and serum (p<

								0.001). In addition, the level of neu protein in oral mucosal cells correlated with the serum neu protein concentration in patients with oral leukoplakia before treatment (p< 0.001).
Uehara et al. 2010 Japan Follow up: NA	**mean nuclear variation**	tissue (biopsy)	7	NA	NA	8/12	Leukoplakia samples: epithelial dysplasias	Leukoplakia samples: 4 tongue 1 hard palate 1 soft palate 1 buccal mucosa	The mean nuclear areas: Before PDT: range: 54.1 to 158.8 µm2 (median=91.4 µm2, SD=23.0 µm2); After PDT: range: 24.6 to 106.8 µm2 (median=66.5 µm2, SD=21.7 µm2; p=0.0920). Significant differences

									in PCNA LI values before and after PDT were observed in: Recurrent grp (p=0.0496); non-recurrent grp (p =0.0188) with decreased PCNA LI values after PDT).
Cell signaling									
Saintigny et al. 2011 USA Follow up: median of 7.5 years	**proteasome machinery, MYC ribosomal components**	tissue (biopsy)	35	Smokers: 22 current 35 former 29 never Alcohol use: 49 current 8 former 29 never	median: 57.5 range: (23-90)	45/41	32 dysplasia 54 hyperplasia	NA	Gene sets associated with proteasome machinery, MYC upregulation as well as ribosomal components were associated with a high risk to develop OSCC.
Saintigny et al. 2018 USA	**MET**	tissue (biopsy)	35 (pts who developed cancer)	Smokers: 42 current 45 former 33 never	NA	61/59	44 dysplasia 76 hyperplasia	NA	No MET expression: Leukoplakia cases (26/120; 21.7%). MET expression:

Follow up: The median follow-up of the 51 patients who did not develop OSCC was 6.08 years			Alcohol use: 70 current 10 former 40 never			low (score 1), 34 (28.3% mild (score 2): 39(32.5%) high (score 3) 21(17.5%). Time-to-cancer-progression varied across 4 Met expression grps (score 0 through 3), p=0.001). Oral cancer–free survival - decreased in patients with high MET expression levels (p<0.001). MET overexpression in leukoplakia: HR: 3.84 (95%CI: 1.59-9.27, p<0.003) for malignant transformation.

| Sakata et al. 2017 Japan Follow up: NA | **SMAD4** | tissue (biopsy) | 127 (leukoplakias that did not transform) | NA | median: 61.7 range: (19-87) | 67/60 | Cases: 13 no dysplasia 7 mild dysplasia 3 moderate/ severe dysplasia Controls: 88 no dysplasia 30 mild dysplasia 9 moderate/ severe dysplasia | 64 (42.7%) gingiva 52 (34.7%) tongue 21 (14.0%) buccal mucosa 13 (8.7%) other oral cavity sites | No. with malignant transformation: 23/150 (15.3%). Malignant transformation associated with: SMAD4 expression (p=0.0017) -lymphocyte infiltration (p= 0.0054). Low SMAD4 expression: - significant prognostic factor in pts with leukoplakia (hazard ratio, 2.632; p=0.043) -correlated with high lymphocyte infiltration (p=0.00035), -significant correlation between |

									low SMAD4 expression and high lymphocyte infiltration (p=0.00027).
Insensitivity to antigrowth signals									
Cell cycle regulators									
Lee et al. 2000 USA Follow up: 7 years (range: 0.2– 10.6)	**p53** **RAR-β** **CP** **LOH** **micro-nuclei**	tissue (biopsy)	70	Smokers: 39 current 31 former/ never Tobacco chewers: 10 yes/60 no Alcohol use: 49 current 21 former/ never Cancer history: 11 yes/59 no	NA	33/37	9 moderate/ severe dysplasias 61 hyperplasia/ mild dysplasia	27 tongue/FOM 43 others	31.4% of patients developed OSCC. High chromosomal polysomy (CP) expression, p53 protein accumulation in the parabasal layer, and LOH at chromosome 3p or 9p: associated with higher cancer risk (p=0.0008).
Nagao et al. 2017	**p53** **ki67**	tissue (biopsy)	4	Smokers: 1 former	median: 66	2/2	Responders: dysplasia (1/4)	Responders: tongue/FOM	p53 expression: greater in basal layers

Japan Follow up: NA				3 never Alcohol use: 2 regular 2 never			no dysplasia (3/4) <u>Non-responders:</u> dysplasia (4/12) no dysplasia (5/12)	(4/4); <u>Non-responders:</u> (7/12), other (5/12)	vs. para-basal layers. Mean para-basal labeling index (LI) of p53 was higher in non-responders (26.0) than in responders (11.2) (p=0.028). Ki67 LIs were similar between groups.
Tanić et al. 2009 Serbia Follow up: 4 years	**p53**	<u>Cases:</u> tissue (biopsy) <u>Controls:</u> blood	32	All smokers	NA	NA/NA	30 mild/ no dysplasia 32 moderate/ severe dysplasia	NA	p53 gene was mutated in 13 cases (40.6%). The majority of mutations were localized on exon 6. Four patients with moderate instability and mutated p53 underwent malignant transformation.
Visioli et al. 2012 Brazil	**p53** **p21WAF1**	tissue (biopsy)	36	NA	Mean: 50.1 SD (±14.5)	19/17	NA	11 gingiva 10 tongue 8 buccal mucosa	Overexpression of p53 and p21WAF1: observed in dysplastic and non-dysplastic

Follow up: 3-6 years								6 lip 1 palate	leukoplakias.
Loss of heterozygosity									
Mao et al. 1996 USA Follow up: median time to OSCC progression: 63 months (range: 5-92)	**markers located at chromosomes 9p21 & 3p14**	tissue (biopsy)	37	22 smokers	57.5	20/17	32 dysplasia 52 hyperplasia and/or hyper-keratosis	NA	8/37(22%) developed OSCC. LOH: (19/37) (51%). -Cases (37%) with LOH at either 9p21 or 3p14 (or both): developed cancer -One case (6%) without LOH underwent malignant transformation. Time to cancer development was shorter in LOH (p=0.039).
Ion channels									
Fernandez-Valle et al. 2016 (A) Spain	**Kv3.4**	tissue (biopsy)	62	28 smokers (45%) of whom 14 (22.6%) also consumed	61.1 SD: +12.41 Range: (30-85)	35/27	43 hyperplasia 8 mild dysplasia 7 moderate dysplasia 4 severe	Cannot discriminate between leukoplakia and OSCC	Detection of Kv3.4+ immunostaining noted in: -hyperplastic leukoplakias 7/ 43

Follow up: median time to OSCC progression: 25.5 months (range: 7-308).				alcohol			dysplasia/ carcinoma in situ		(16%) -leukoplakic lesions with dysplasia 8/16 (50%) (p=0.008). No correlation observed between Kv3.4 expression in oral leukoplakia compared to OSCC samples (p=0.86). Leukoplakias with Kv3.4 neg expression: 8/13 (61.5%) showed strong Kv3.4 immunostaining in after subsequent OSCC development
Fernández-Valle et al. 2016 (B) Spain Follow up: median time	**HERG1 protein expression**	tissue (biopsy)	62	28 smokers (45%) of whom 14 (22.6%) also consumed alcohol	Mean 61.1 SD ±12.4 Range (30–85)	27/35	43 hyperplasia 8 mild dysplasia 7 moderate dysplasia 4 severe dysplasia/	NA	HERG1 status of lesions on OSCC development: -HERG1-(+): 8/22 (36%); HERG1(-neg): 18/39 (46%)

to OSCC progression: 39 months (range: 7 - 226)						carcinoma in situ		

Sustained angiogenesis

Growth factors

Nayak et al. 2015 India Follow up: median time to OSCC progression: 29.41 months	*FGF-2* *FGFR-2* *FGFR-3*	tissue (biopsy)	43	NA	NA	NA/NA	NA	NA	Expression of FGF-2 and FGFR-2: associated with HR 28.2 (95% CI: 3.29–241.72; p<0.002) and HR 11.5 (95% CI: 2.20–59.91; p<0.004) of cancer progression, respectively.
Yang CZ et al. 2014 NA country Follow up: up to 5 yrs	**GDF15**	serum	24	NA	NA	NA/NA	NA	NA	Serum GDF15 level: increased leukoplakia and OSCC cases compared with healthy controls (p< 0.001). OSCC cases

									with GDF15 <346.9 ng/L had improved 3-year disease-free survival rate compared to cases withGDF15 $\geq$ 346.9 ng/L. (64.3% vs 56.5%)
Podoplanin									
Kawaguchi et al. 2008 USA Follow up: 7.5 years	**podoplanin**	tissue (biopsy)	163	Smokers: 52 current 60 former Alcohol use: 85 current 18 former	NA	NA/NA	101 hyperplasias 49 dysplasias	NA	Podoplanin (+) lesions: more common in older patients (p=0.016), females (p= 0.02), and those with dysplastic leukoplakias (p=0.04). 23% of the patients progressed to OSCC. Podoplanin was an independent risk factor for malignant transformation (HR=3.087; 95% CI, 1.530-6.231; p= 0.002).

Saintigny et al. 2009 USA Follow up: median of 7.5 years	**ΔNp63 podoplonin**	tissue (biopsy)	162	Smokers: 56 current 65 former 41 never Alcohol use: 93 current 19 former 50 never	NA	85/77	NA	NA	Oral cancer was associated with: -ΔNp63 and podoplanin status (p< 0.0001), -ΔNp63 status and intraepithelial inflammatory cell clusters (EIC) (p= 0.0003), -between podoplanin status and presence of EIC (p=0.0012). Presence of all three biomarkers was associated with dysplastic histology (p= 0.016) and older age (p=0.004). Cumulative incidence for developing oral cancer at 3 years of follow-up: 53% for

								cases with all three biomarkers compared to 11% of other patients. Patients with podoplanin-positive leukoplakia and those with ΔNp63-positive leukoplakia had a higher incidence of OSCC (p<0.0001).

Glucose regulated proteins

Lin et al. 2010 Taiwan Follow up: median of 5.4 years (range: 0.02–6.8)	**GRP-78**	tissue (biopsy)	28	NA	NA	NA/NA	NA	Over-expression of GRP78 was associated with increasing malignant potential in 14% of leukoplakia patients. Patients with GRP78 over-expression had a poorer 6-year same-site premalignancy-free survival rate than those without (44.0%

| | | | | | | | | | vs 89.6%, respectively; p<0.002). |

Gene transcription modification

miRNA expression

Xiao et al. 2012 China Follow up: median of 32 months (range: 20-47)	**miRNA**	tissue (biopsy)	7 (progressing leukoplakias)	Smokers: 3 current 4 never/former Alcohol use: 4 current 3 never/former	Mean: 55.4 SD±16.3	3/4	Non-transformed leukoplakias: 11 no/mild dysplasia 5 moderate dysplasia 4 severe dysplasia	NA	Leukoplakia cases who OSCC had -upregulation of: miR-31, miR-142-5p, miR-33a, miR-1259, miR-146b-5p, miR-886-3p, miR-886-5p, miR-519d, and miR-301a - downregulation of: miR-572, miR-611, miR-602, miR-675, miR- 585, miR-623, miR-637, and miR-1184. FISH analysis confirmed high miR-31 expression in leukoplakia that underwent malignant

									transformation.
Yang Y et al. 2013 China Follow up: median time of 35 months (range: 26-46)	**miRNA**	saliva	15	<u>Smokers:</u> 7 current 3 former 5 never/former	Mean 57.6	3/4	All 15 samples were LGD (mild to moderate dys-plasia). Among those that progressed 5 were severe dysplasia (3/5 with SCC micro-foci), 2 CIS, 1 moderate to severe dysplasia SCC micro-foci	5 lateral tongue 2 lateral palate 6 buccal mucosa 1 palate 1 lower lip	There was up-regulation of miR-708, miR-10b, miR-19a, miR-30e, miR-26a, miR-660 and down-regulation of miR-99, miR-15a, miR-197, miR-145 and miR-150. miRNAs were differentially expressed in non-progressing low grade dysplasias with different progression potentials. miR-181c and miR-181b were under-expressed in progressing low grade dysplasias and over-expressed in non-progressive low grade dysplasias.

DNA methylation									
Foy et al. 2015 USA Follow up: For the 12 patients who did not develop OSCC (median follow-up, 7.64 years) and the patients who did develop OSCC (group B, median follow-up, 2.15 years)	**DNA methylation**	tissue (biopsy)	24	NA	NA	NA/NA	Reported in the supplementary material	NA	86 candidate genes were identified. Methylation of *PENK*, *FOXI2*, and *AGTR1* promoter regions were associated with the development of OSCC.

* **Please see Table 4 for explanation of biomarkers**;

**Some studies reported the median and not the mean;

#: number; CIS: carcinoma in situ; F: females; FOM: floor or the mouth; leukoplakia (n): leukoplakia group sample size; LOH: loss of heterozygosity; M: males; NA: not available; OSCC: oral squamous cell carcinoma; PDT: photodynamic therapy; pt(s): patient(s); SCC: squamous cell carcinoma y: year(s).

Table 3. Quality assessment of the studies according to the Quality in Prognosis Studies (QUIPS) criteria (Hayden _et a.l,_ 2013), illustrated in Figure 2.

Author	Year	Study participation	Study attrition	Prognostic factor measurement	Outcome measurement	Study confounding	Statistical analysis and reporting
Beenken S. _et al._	1994	high	moderate	moderate	low	moderate	moderate
Beenken S. _et al._	1999	high	moderate	moderate	low	moderate	moderate
Chang P. _et al._	2013	moderate	moderate	high	moderate	moderate	low
Fernandez-Valle Á. _et al._ (A)	2016	low	low	low	low	low	low
Fernández-Valle Á. _et al._ (B)	2016	moderate	low	low	moderate	moderate	low
Foy JP. _et al._	2015	low	low	low	low	moderate	low
Kawaguchi H. _et al._	2008	low	low	low	moderate	moderate	low

Lee JJ. *et al.*	2000	low	low	low	low	moderate	low
Lin CY. *et al.*	2010	low	moderate	moderate	moderate	high	low
Mao L. *et al.*	1996	low	low	low	moderate	low	low
Massarelli E. *et al.*	2005	low	low	moderate	low	low	low
Nagao T. *et al.*	2017	moderate	low	moderate	moderate	moderate	moderate
Nayak S. *et al.*	2015	low	moderate	moderate	moderate	moderate	low
Rai B. *et al.*	2010	moderate	moderate	moderate	moderate	moderate	moderate
Saintigny P. *et al.*	2009	low	low	low	low	moderate	low
Saintigny P. *et al.*	2011	low	low	low	low	low	low
Saintigny P. *et al.*	2018	low	low	low	low	moderate	low
Sakata J. *et al.*	2017	low	low	low	low	low	low
Tanić N. *et al.*	2009	low	low	low	low	moderate	low
Uehara M. *et al.*	2010	low	moderate	moderate	moderate	moderate	low
Visioli F.	2012	low	low	moderate	moderate	moderate	low
Wan XS.	1999	low	moderate	moderate	moderate	moderate	moderate
Xiao W	2012	low	moderate	low	moderate	moderate	low
Yang CZ	2014	moderate	moderate	low	moderate	moderate	low
Yang Y	2013	moderate	moderate	moderate	low	moderate	low

Table 4. Biomarkers used with leukoplakia specimens in the 25 studies displayed in Table 2.

Biomarker Acronym	Biomarker Name & Function
CP	chromosomal polysomy;
CRP	C-reactive protein; acute inflammatory marker
E-cadherin	molecule that makes cells adhere to each other (cadherin="calcium-dependent adhesion")
eGFR, EGFR	estimated glomerular filtration rate based on blood creatinine level
E-selectin	cell adhesion molecule only expressed on endothelial cells activated by cytokines; inflammatory marker
FGF	basic fibroblast growth factor and signaling protein
FGFR	fibroblast growth factor receptor
GDF	growth differentiation factor
GRP	glucose-regulated protein
HER	human epidermal growth factor receptor
HERG	human ether-à-go-go related gene
ICAM	intercellular adhesion molecule; inflammatory marker
IL	interleukin, cytokines (secreted proteins, signal molecules)
Ki-67	antigen, nuclear protein
Kv3.4	potassium voltage-gated channel gene
M-CSF	macrophage colony-stimulating factor; inflammatory marker
MET	mesenchymal epithelial transition
MDA	malonaldehyde; oxidative marker
miRNA	micro-**ri**bo**n**ucleic **a**cid
MMP	matrix metalloproteinase
MYC	family of regulator genes and proto-oncogenes that code for transcription factors (MyC: **My**elo**c**ytomatosis)
ΔNp63	Tumor protein p63

8-OHdG	8-hydroxydeoxyguanosine
p21WAF1	tumor protein, cell cycle protein regulators
p27	protein that regulates the cell cycle
p53	tumor protein, cell cycle protein regulators
podoplanin	mucin-type protein, specific lymphatic vessel marker
RAR-β	retinoic acid receptor-Beta
SAA	serum amyloid A; inflammatory marker
SMAD4	Mothers against decapentaplegic homolog 4 gene
TGF	transforming growth factor; inflammatory marker

FIGURE LEGENDS

Figure 1.

Selection of studies for systematic review of prognostic biomarkers for oral leukoplakia.

Figure 2.

Risk of bias in the 25 included studies according to the Quality in Prognosis Studies (QUIPS) criteria (Hayden *et al.*, 2013).

Supplementary Results

Identified biomarkers

Tissue invasion, metastasis, and inflammatory markers

In a prospective longitudinal study, Chang *et al.* examined a panel of inflammatory biomarkers (interleukin-6 [IL-6], macrophage colony-stimulating factor [M-CSF], transforming growth factor beta [TGF-β1], intercellular adhesion molecule 1 [ICAM-1],
E-selectin, C-reactive protein [CRP], serum amyloid A [SAA], matrix metalloproteinase-2 [MMP-2], MMP-9) in patients with OSCC (n=46), normal tissue from healthy controls (n=111), and oral leukoplakia patients (n=46) (Chang *et al.*, 2013). Patients with leukoplakia underwent biopsy and serum sample collection at the first visit. Serum samples were collected from healthy controls and OSCC patients (prior to surgery and 1, 2, 3, 6, 12, 18, and 24 months after surgery). Enzyme-linked immunosorbent assay (ELISA) was utilized for serum inflammatory marker detection.

When leukoplakia serum marker levels were considered, there were statistically significant differences for TGF-β1 (AUC: 0.756, p<0.001), CRP (AUC: 0.704, p<0.001) and MMP-9 (AUC: 0.729, p<0.001) compared to healthy controls. Sensitivity for presence of leukoplakia or OSCC following evaluation of MMP-9, MMP-2, TGF-β1, CRP, and E-selectin was 67.4% and 80%, respectively (serum markers). No detailed follow-up or malignant progression data were reported in this study.

In a study by Massarelli's group, immunohistochemical (IHC) analysis was used to assess E-cadherin expression in 31 patients with oral leukoplakia and 15 with laryngeal leukoplakia (Massarelli et al., 2005b). Further, 40 of 46 samples were additionally analyzed for p27

expression. All patients were included in 3 chemoprevention trials. Loss of E-cadherin expression and development of OSCC after a median of 21 months occurred in 25 of 46 patients (54%) and 14 of 23 patients (61%), respectively. Fourteen of 31 patients (45%) with oral leukoplakia developed OSCC. The frequency of the loss of membranous E-cadherin increased with histologic progression. Thirteen of 19 patients (68%) who had p27 loss, and 7 of 21 patients (33%) who had lesions with p27 retention developed OSCC. Multivariate analysis showed that p27 loss was an independent predictor of malignant transformation (log-rank test; p=0.02). The combination of E-cadherin and p27 loss was associated with significantly decreased time to progression to cancer (log-rank test; p=0.04).

Serum oxidative markers

Rai *et al.* measured salivary and serum oxidative markers including malonaldehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), vitamins C and E before curcumin caplets intake, one week after curcumin administration started, and once the oral potentially malignant lesions resolved completed (Rai *et al.*, 2010). Clinical and histopathological examinations were conducted as well prior to intake of curcumin. One hundred patients were included in this study (25 with oral leukoplakia, 25 with oral submucous fibrosis (OSMF), 25 with oral lichen planus, 25 healthy individuals). MDA and 8-OHdG levels were significantly higher in oral leukoplakia, OSMF, and oral lichen planus patients, when compared with healthy controls (p<0.05), while serum and salivary vitamin C and E levels were significantly lower (p<0.05). Median salivary and serum MDA, 8-OHdG, and vitamin C and E levels were significantly different in patients with oral leukoplakia, OSMF and oral lichen planus before the intake of curcumin and after treatment (p<0.05).

Proliferation without exogenous stimulation

Growth factors

Two studies investigated the expression of epidermal growth factor receptor (EGFR) and transforming growth factor alpha (TGF-α) in leukoplakia (Lee et al., 2000, Beenken et al., 1994). Of note, both studies were part of a chemoprevention trial with 13-cis-retinoic acid therapy. In the first work (Beenken *et al.*, 1994), IHC was performed to evaluate the expression EGFR, TGF-a, HER-2/neu in nine leukoplakia cases and surrounding normal-appearing mucosa.

Pretreatment expression scores for EGFR, TGF-α, and HER-2/neu in leukoplakia were increased when compared to their expression scores in normal-appearing mucosa. TGF-α expression ratios in leukoplakia were significantly reduced after treatment with 13-cis-retinoic acid. In the follow-up study (Beenken *et al.,* 1999), IHC was used to evaluate TGF-α and EGFR expression in eleven tissue samples obtained from oral leukoplakia lesions and compared to adjacent normal mucosa and the oral mucosa from three healthy volunteers. TGF-α and EGFR scores were increased in dysplastic oral leukoplakia specimens when compared with adjacent normal appearing mucosa. TGF-α expression scores in normal-appearing mucosa adjacent to dysplastic tissue were higher than those seen in the oral mucosa from healthy volunteers. Neither study presented any additional information on the malignant progression of the leukoplakias.

Another study was conducted to explore the relationship between protease activity and neu protein levels in patients with oral leukoplakia (n=25) enrolled in a chemoprevention trial with Bowman-Birk inhibitor concentrate(Wan *et al.*, 1999). Changes in protease activity in oral mucosal cells after administration of the chemopreventive agent correlated with the changes in the neu protein levels in oral mucosal cells ($p<0.001$) and serum ($p<0.001$). In addition, the level of neu protein in oral mucosal cells correlated with the serum neu protein concentration in patients with oral leukoplakia before treatment ($p<0.001$). This correlation, however, was not observed after receiving further treatment with the chemopreventive agent.

Uehara's group analyzed the morphological changes of cell nuclei and proliferating activity of 14 OSCCs, one verrucous carcinoma, and 7 leukoplakias (epithelial dysplasias) before and after photodynamic therapy (PDT) in order to predict recurrence(Uehara *et al.*, 2010). The mean nuclear area (NA) and coefficient of variation of the nuclear area (NACV) of 100 nuclei per slide were calculated using computer-assisted image analysis in tissue specimens before and after PDT. In addition, proliferating cell nuclear antigen (PCNA) IHC was performed in each specimen. The mean NAs before PDT ranged from 54.1 to 158.8 μm^2 (median=91.4 μm^2, SD=23.0 μm^2), and those after PDT from 24.6 to 106.8 μm^2 (median=66.5 μm^2, SD=21.7 μm^2; p=0.092). Significant differences in PCNA Labelling Index (LI) values before and after PDT were observed in both the recurrent (p=0.050) and non-recurrent (p=0.019) groups (decreased PCNA LI values after PDT).

Cell signaling

Saintigny and colleagues performed gene expression profiling in patients with oral leukoplakia (n=86) and found that gene sets associated with proteasome machinery, MYC oncogene upregulation as well as ribosomal components were associated with a high risk for developing oral cancer (n=35; median follow-up: 7.5 years) (Saintigny *et al.*, 2011). In 2018, the same authors evaluated the mesenchymal epithelial transition (MET) receptor tyrosine kinase expression using IHC in the same group of leukoplakia patients (n=86, 35 patients who developed oral cancer and 51 who did not develop OSCC, randomly selected from among the 162 participants of the original chemoprevention trial), and its association with cancer development (Saintigny *et al.*, 2018). Only 120 individuals had available material. No MET expression was observed in 26 leukoplakias (26/120; 21.7%). MET was expressed at low (score 1), mild (score 2), and high (score 3) levels in 34 (28.3%), 39 (32.5%), and 21 (17.5%) patients, respectively. Time-to-cancer-progression varied significantly in the four MET expression groups (MET expression score 0 *vs.* score 1 *vs.* score 2 *vs.* score 3; log-rank test, p=0.001). Oral cancer– free survival was significantly decreased in patients with high MET expression levels (p<0.001). MET overexpression in leukoplakia was associated with a significant hazard ratio of 3.84 (95%CI: 1.59-9.27, p<0.003) for malignant transformation.

Sakata and colleagues performed IHC to measure transcription factor protein SMAD4 (Mothers against decapentaplegic homolog 4) expression in oral leukoplakia (n=150) and OSCC tissue sections (n=36). In total, 23 patients (23/150; 15.3%) underwent malignant transformation (Sakata *et al.*, 2017). Malignant transformation was significantly associated with the status of SMAD4 expression (p=0.0017) and lymphocyte infiltration (p=0.0054). Low SMAD4 expression was a significant prognostic factor in leukoplakia patients (hazard ratio, 2.632; p=0.043). In addition, low SMAD4 expression was closely correlated with high lymphocyte infiltration (p=0.00035), resulting in a significant correlation between the combination of low SMAD4 expression and high lymphocyte infiltration with malignant transformation of leukoplakia (p=0.00027). No significant associations of malignant transformation were observed when age, gender, site, or dysplasia grade were considered.

Insensitivity to antigrowth signals

Cell cycle regulators

In a chemoprevention trial with isotretinoin, Lee and colleagues (Lee *et al.*, 2000) investigated the molecular-cellular factors associated with risk of progression to OSCC in patients with oral leukoplakia (n=70). Over a median of seven years (range: 0.2–10.6), 31.4% of patients developed OSCC. High chromosomal polysomy (CP) expression, p53 protein accumulation in the parabasal layer, and loss of heterozygosity (LOH) at 3p or 9p were associated with higher cancer risk (p=0.0008).

In a separate chemoprevention trial observing patients treated with low doses of beta-carotene and vitamin C supplements, oral leukoplakia specimens collected from the participants (4 responders and 12 non-responders) were examined for p53 and Ki67 expression, and the percentage of positive cell nuclei were analyzed using LI (Nagao *et al.*, 2017). p53 expression was greater in basal layers when compared to para-basal layers. The mean para-basal LI of p53 was higher in non-responding (26.0) than in responding patients (11.2) (p=0.028), whereas Ki67 LIs were similar in the two groups.

Tanić*'s* team analyzed genomic instability by comparing the DNA fingerprints of 32 leukoplakias with paired normal tissue (Tanic *et al.*, 2009). In addition, mutational status of the p53 gene was analyzed using PCR–single-stranded conformational polymorphism (SSCP), PCR–heteroduplex DNA and DNA sequencing. The p53 gene was mutated in 13 cases (40.6% of patients) and the majority of mutations were localized on exon 6. Four patients with moderate instability and mutated p53 transformed into OSCC.

Visioli and colleagues assessed p53 and p21WAF1 expression in 36 leukoplakias (Visioli *et al.*, 2012). Overexpression of p53 and p21WAF1 was observed in both dysplastic and non-dysplastic leukoplakias. There were no statistically significant differences among the different types of dysplasia.

Loss of heterozygosity

Mao's group performed a PCR-based microsatellite analysis at chromosomes 9p21 and 3p14 to investigate LOH in a group of patients affected by oral leukoplakia in 84 biopsy samples obtained from 37 patients enrolled in a chemoprevention trial (n=70) (Mao *et al.*, 1996). A total of eight patients underwent malignant transformation (21.6%; 8/37) over a median time of 63 months (range: 5 - 92). LOH was identified in 51% of patients (19/37). Thirty-seven percent of patients with LOH at either 9p21 or 3p14 or both developed cancer while only one patient (6%) without

LOH underwent malignant transformation. Time-to-cancer-development was significantly shorter in the group with LOH (p=0.039).

Ion channels

Fernandez-Valle *et al.* collected oral leukoplakia (n=62) specimens prospectively and OSCC specimens (n=100) retrospectively and assessed them for expression of voltage-dependent potassium channel Kv3.4 and HERG1 (ether-à-go-go related gene 1; also known as KCNH2) by IHC (Fernandez-Valle *et al.*, 2016a, Fernandez-Valle *et al.*, 2016b) .

The main aim of the first study was to investigate the clinical significance of Kv3.4 in the malignant progression of oral leukoplakia (Fernandez-Valle *et al.*, 2016a). This ion channel is thought to play a role in shortening of action potential durations and modulating pre-synaptic neurotransmitter release. Positive Kv3.4 immunostaining was detected in seven of 43 (16%) non-dysplastic leukoplakias and eight of 16 (50%) leukoplakic lesions with dysplasia (p=0.008). No correlation was observed between Kv3.4 expression in oral leukoplakia compared to OSCC samples (p=0.86). Eight (61.5%) of 13 leukoplakias with negative Kv3.4 expression showed strong Kv3.4 immunostaining in the corresponding OSCC that developed subsequently. The median time to cancer progression was 25.5 months (range: 7 - 308). The overall malignant transformation was 43.6%. Patients with dysplasia had an increased risk of malignant transformation compared to those with hyperplasia (p= 0.009). Patients carrying Kv3.4-positive lesions had higher progression risk than Kv3.4-negative lesions (p= 0.58) (Fernandez-Valle *et al.*, 2016a).

In a subsequent study, Fernández-Valle *et al.*, (Fernandez-Valle *et al.*, 2016b) assessed HERG1, which is often overexpressed in neoplastic cell lines and human primary cancers of different histogenesis (D'Amico *et al.*, 2013). They showed that eight of 22 patients (36%) with HERG1-positive leukoplakias developed OSCC, compared to 18 of 39 patients (46%) with HERG1-negative lesions. The median time to cancer diagnosis in leukoplakia patients who underwent malignant transformation was 39 months (range: 7-226).

Sustained angiogenesis

Growth factors

Nayak *et al.*, performed IHC using antibodies against Fibroblast Growth Factor (FGF2) and its receptors FGFR-2 and FGFR-3 in patients with OSMF and leukoplakia (Nayak *et al.*, 2015). Expression of FGF-2 and FGFR-2 was associated with a 28.2-fold (95% CI: 3.29–241.72; p<0.002) and 11.5-fold (95% CI: 2.20–59.91; p<0.004) increased risk of cancer progression, respectively.

Pretreatment serum GDF15 (Growth/differentiation factor 15) concentration was detected using an ELISA in 30 healthy individuals, 24 patients with oral leukoplakia and 60 patients with OSCC (Yang *et al.*, 2014). GDF15 concentration was measured at follow-up visits as well as for OSCC patients (every two months in the first year, every three months in the second year, and every four to six months thereafter for a maximum of five years). Serum GDF15 concentration was significantly higher in patients with oral leukoplakia and OSCC, compared with healthy controls (p< 0.001). OSCC patients with serum GDF15 concentration <346.9 ng/L had an improved 3-year disease-free survival rate (64.3% vs 56.5%) compared with those above 346.9 ng/L.

Podoplanin

Kawaguchi and team followed 163 patients for 7.5 years and IHC was performed to assess podoplanin (a mucin-type transmembrane protein) expression in the epithelium in a prospective intervention study of 150 patients randomly assigned to intervention with 13-cis-retinoic acid versus carotene retinyl palmitate versus retinyl palmitate alone (Kawaguchi *et al.*, 2008). Podoplanin-positive lesions were significantly more common in older patients (p=0.016), females (p= 0.02), and patients with dysplastic leukoplakias (p=0.04). Twenty-three percent of the patients progressed to OSCC. Podoplanin was determined to be an independent factor for malignant transformation in the multivariate analysis (hazard ratio=3.087; 95% CI, 1.530-6.231; p= 0.002).

A separate study assessed whether overexpression of ΔNp63 alone or in combination with other molecular and morphologic features was associated with a high risk of malignant transformation in 152 patients with oral leukoplakia enrolled in a chemoprevention trial (Saintigny *et al.*, 2009). Fifty-six (37%) were classified as podoplanin positive, and 94 (63%) were negative. Podoplanin expression was more frequent in older patients, females and dysplastic tissues. Expression of ΔNp63 was more frequent in females (p=0.02) and in white patients (p=0.04). There was a significant association between ΔNp63 and podoplanin status (p<0.0001), between ΔNp63 status and the presence of clusters of intraepithelial inflammatory cells (EIC)

(p=0.0003), and between podoplanin status and the presence of EIC (p=0.0012). The presence of all three positive biomarkers was associated with dysplastic histology (p=0.016) and older age (p=0.004). The cumulative incidence rate for developing oral cancer at 3 years of follow-up was 53% for patients with all three positive biomarkers compared with 11% of the other patients. Patients with podoplanin-positive leukoplakia as well as those with ΔNp63-positive leukoplakia had a significantly higher incidence of OSCC (p<0.0001). At 5 years, the OSCC–free survival rate for patients with ΔNp63-negative leukoplakia was 89% (95% CI: 0.83-0.95) compared with 61% for patients with ΔNp63-positive leukoplakia (95% CI: 0.47-0.78). The malignant transformation risk was lower when considering the presence of clusters of EIC, but was statistically significant (p=0.015). At 5 years follow-up, the OSCC–free survival rate for patients with no EIC clusters was 86% (95% CI: 0.79-0.93) compared with 72% for patients with EIC clusters.

Glucose regulated proteins

Lin and colleagues investigated the expression levels of GRP78 in OPMDs and examined whether it could act as an independent predictive factor for malignant transformation (Lin *et al.*, 2010). GRP78 belongs to the HSP70 protein family, which plays critical roles in the stress of oncogenesis. Two hundred and four patients with OSCC and 86 with OPMDs (and normal tissue from the same patient) were included. GRP78 expression was assessed using western blot analysis. Patients with OPMDs were followed for a median of 5.4 years (range: 0.02–6.83). Over-expression of GRP78 was associated with increasing malignant potential in 14% of leukoplakia patients, 27% of those with erythroplakia, 50% with verrucous leukoplakia, and 74% with OSCC (p<0.0001). Patients with GRP78 over-expression had a poorer 6-year same-site premalignancy-free survival rate than those without (44.0% vs 89.6%, respectively; p<0.002).

<u>Gene transcription modification</u>

miRNA expression

Two studies focused on miRNA expression as possible biomarkers for progression in patients affected by oral leukoplakia. Xiao *et al.* compared miRNA expression in a group of patients with oral leukoplakia that did not progress to cancer (n=20) and a group of patients whose leukoplakias underwent malignant transformation (n=7; 25.9% (7/27)) (Xiao *et al.*, 2012). Patients with leukoplakia that transformed into OSCC had: 1) upregulation of miR-31*, miR-142-

5p, miR-33a, miR-1259, miR-146b-5p, miR- 886-3p, miR-886-5p, miR-519d, and miR-301a; and 2) downregulation of miR-572, miR-611, miR-602, miR-675, miR- 585, miR-623, miR-637, and miR-1184. Fluorescence in situ hybridization (FISH) analysis confirmed that miR-31* was highly expressed in leukoplakia that underwent malignant transformation.

Yang *et al.* performed real-time qPCR on saliva samples of patients with progressing leukoplakias (n=7) with low grade dysplasia and non-progressing low grade dysplasias (n=8) enrolled in a clinical trial and received 13-cis-retinoic acid to explore possible differences in microRNA regulation (Yang *et al.*, 2013). Progression was defined as development of 86 candidate geneshigh-grade dysplasia, carcinoma *in situ* or OSCC. Patients were followed for a median of 35 months (range: 26 - 46). Overall, there was up-regulation of miR-708, miR-10b, miR-19a, miR-30e, miR-26a, miR-660 and down-regulation of miR-99, miR-15a, miR-197, miR-145 and miR-150. miRNAs were differentially expressed in non-progressing low grade dysplasias with different progression potentials. miR-181c and miR-181b were under-expressed in progressing low grade dysplasias and over-expressed in non-progressive low grade dysplasias.
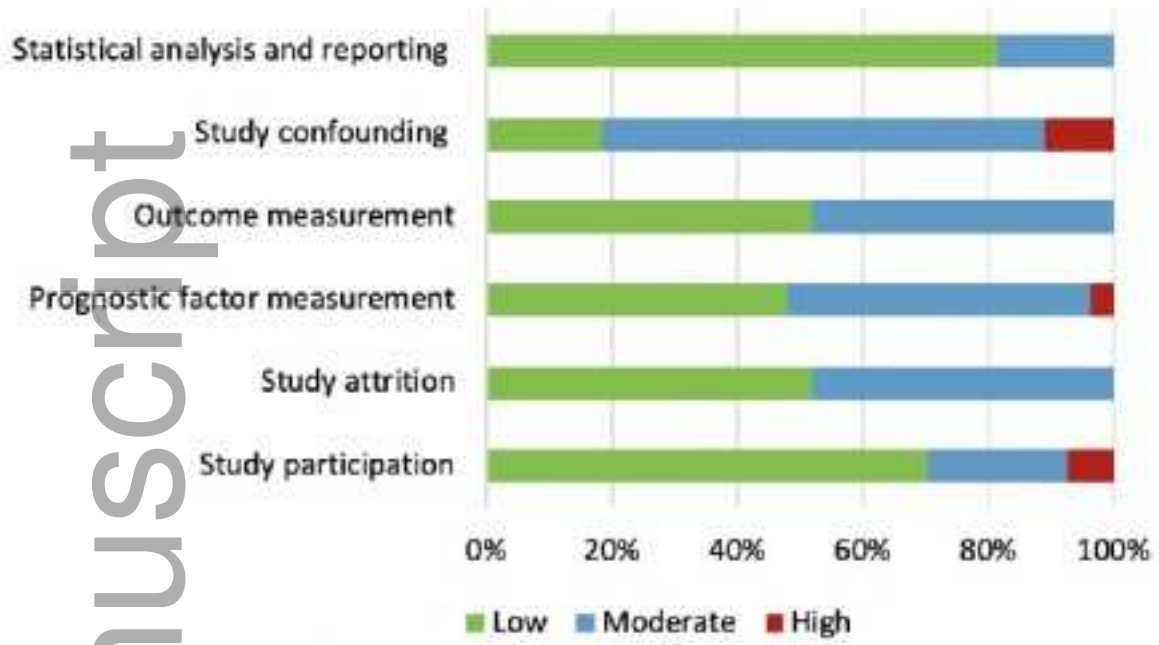
DNA methylation

Foy *et al.* detected DNA methylation changes in leukoplakia patients (n=12; median follow-up: 2.15 years) who developed OSCC, compared with leukoplakia patients (n=12; median follow-up: 7.64 years) who did not develop OSCC (Foy *et al.*, 2015). The most frequent 86 genes differentially methylated between patients who did and did not develop OSCC were simultaneously hypermethylated. Promoter methylation of *PENK*, *FOXI2*, and *AGTR1* were associated with the development of OSCC. The majority of the 86 genes were non-methylated in normal tissues and hypermethylated in OSCC.
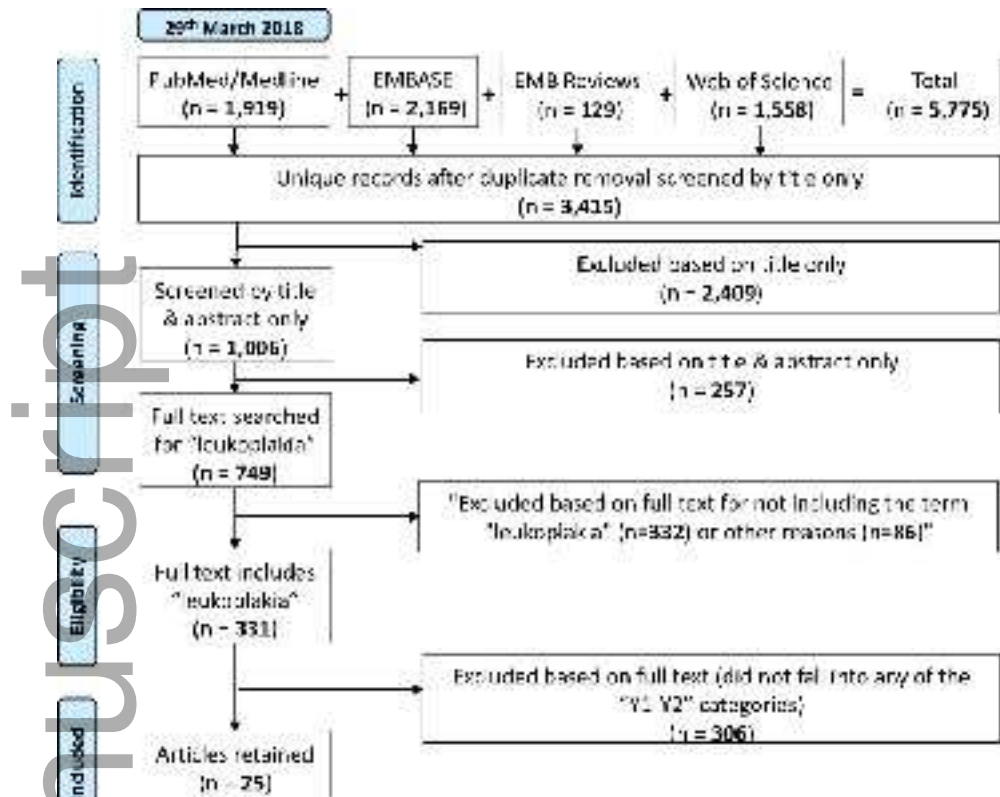
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