DOI:
10.1016/j.oooo.2016.02.008

Document Version
Peer reviewed version

Accepted Manuscript

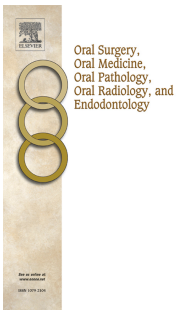

Image-based DNA ploidy analysis aids prediction of malignant transformation in oral lichen planus

Marcelo Sperandio, DDS MSc PhD, Myriam F. Klinikowski, DDS MSc MBBS, Amy L. Brown, BSc(Hons) MBBS, Penelope J. Shirlaw, BDS FDS RCPS, Stephen J. Challacombe, FMedSci DSc, Peter R. Morgan, PhD FRCPath, Saman Warnakulasuriya, FDS PhD DSc, Edward W. Odell, PhD FRCPath

PII: S2212-4403(16)00067-5

DOI: 10.1016/j.oooo.2016.02.008

Reference: OOOO 1433

To appear in: *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*

Received Date: 23 September 2015

Revised Date: 3 January 2016

Accepted Date: 16 February 2016

Please cite this article as: Sperandio M, Klinikowski MF, Brown AL, Shirlaw PJ, Challacombe SJ, Morgan PR, Warnakulasuriya S, Odell EW, Image-based DNA ploidy analysis aids prediction of malignant transformation in oral lichen planus, *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* (2016), doi: 10.1016/j.oooo.2016.02.008.

Title: Image-based DNA ploidy analysis aids prediction of malignant transformation in oral lichen planus

Authors, academic qualifications and affiliations*:* Marcelo Sperandio[a,b] DDS MSc PhD, Myriam F Klinikowski[c] DDS MSc MBBS, Amy L Brown[a,b] BSc(Hons) MBBS, Penelope J Shirlaw[c] BDS FDS RCPS, Stephen J Challacombe[c] FMedSci DSc, Peter R Morgan[a] PhD FRCPath, Saman Warnakulasuriya[c] FDS PhD DSc, Edward W Odell[a] PhD FRCPath

[a] Department of Oral Pathology, King's College London. Guy's Hospital, London SE1 9RT. United Kingdom

[b] Department of Oral Pathology & Medicine, Sao Leopoldo Mandic Dental Institute and Research Center, Rua Jose Rocha Junqueira, 13, Campinas-SP, CEP 13045-755, Brazil

[c] Department of Oral Medicine, King's College London, King's College Hospital, London SE5 9RS. United Kingdom

Corresponding author*:* Marcelo Sperandio, Department of Oral Pathology & Medicine, Sao Leopoldo Mandic Dental Institute and Research Center, Rua Jose Rocha Junqueira, 13, Campinas-SP, CEP 13045-755, Brazil. Telephone: +551932113600. Email: marcelo.sperandio@slmandic.edu.br

Disclosures: The authors declare no conflict of interest.

Statement of Clinical Relevance: there is currently no reliable marker of malignant transformation in oral lichen planus. This study demonstrates that DNA ploidy analysis can predict malignant change in up to 50% of patients with oral lichen planus.

Acknowledgements

We are grateful to The Charitable Foundation of Guy's and St Thomas' Hospitals for funding the study and to CAPES (Brazilian Higher Education Support) for support to MS.

Abstract word count: 197

Manuscript word count: 3,601

Number of references: 48

Number of figures and tables: 4

Abstract

Objectives: To investigate the potential of image-based DNA ploidy analysis to predict malignant transformation in patients with oral lichen planus (OLP).
Study design: DNA ploidy analysis was performed on biopsy samples from 14 patients with OLP who underwent malignant transformation. As controls, 42 OLP lesions showing unusual clinical features suggesting a transformation risk and 68 samples of clinically and histologically typical OLP were included. Cases with dysplasia on initial biopsy were excluded. Eighty fibroepithelial polyps acted as methodological controls. Epithelial nuclei were isolated from formalin-fixed paraffin embedded biopsy samples and monolayers stained with Feulgen for automated image cytometry to establish DNA content. Ploidy status was correlated to outcome using Kaplan-Meier analysis and Log rank Mantel-Cox tests.
Results: All controls and typical OLP were diploid and none underwent malignant transformation in mean follow-up of 14 years (10–18 years). One unusual OLP developed carcinoma and all were diploid. The 14 patients with transformation developed 21 carcinomas. In the 11 patients who had a prior biopsy, 4 were aneuploid.
Conclusion: DNA ploidy analysis predicted malignant transformation in over one third (36.4%) of patients with OLP with a preceding biopsy (n=11). This premalignant nature could not have been diagnosed clinically or by histological dysplasia assessment.

Keywords: oral lichen planus, malignant transformation, oral cancer, DNA ploidy, image-based cytometry

Introduction

Lichen planus is an idiopathic inflammatory mucocutaneous disease, a cell-mediated autoimmune reaction against keratinocytes, extrinsic antigens or their metabolites bound to or expressed by keratinocytes[1]. Oral mucosa is affected in approximately 50% of patients with skin lesions but 25% of all patients have oral lesions only [2]. The prevalence of oral lichen planus (OLP) varies considerably between studies, ranging from 0.02% in India[3] to 2.4% in Europe [4-8] with a likely overall age standardised prevalence of 1.27% [9]. The best estimate for the UK is 1.4% based on clinical examination[10].

Despite decades of controversy[11-12], any temporal or spatial relationship between squamous carcinoma and OLP remains unclear and difficult to investigate[13]. Patients with OLP are at increased risk of developing oral squamous carcinoma[14], but the risk assessed in a recent hospital series of 0.5%[15] probably overstates the risk. This proportion would be inconsistent with the low overall incidence of oral carcinomas, most of which do not develop in lichen planus.

Difficulty arises because there are no completely specific diagnostic features of oral lichen planus and diagnosis requires clinical and histological features[16]. The clinical features overlap with those of lichenoid contact or drug reactions and leukoplakia. The histological features overlap with dysplasia, which often elicits a cell-mediated immune response easily confused with OLP[17].

There is no reliable marker to predict malignant transformation in oral potentially malignant disorders (OPMD) of any type[18-19] whether OLP, oral lichenoid lesions, leukoplakia or other conditions with dysplasia histologically. However, total nuclear DNA content, as measured by image-based DNA ploidy analysis, has shown a good association with transformation in case series and case-control studies[20-21] and in dysplastic lesions[22] and proliferative verrucous leukoplakia[23.] In our recent large scale outcome study in patients with all types of OPMD, DNA ploidy analysis performed at least as well as dysplasia grading and identified transformation risk in mucosa with

mild dysplasia or normal histology[24]. Routine dysplasia grading - currently the accepted standard method in clinical practice - is poorly standardized[18,25] and poorly predictive of malignant transformation in several large-scale studies[26-30]. DNA ploidy analysis has potential to become the reference standard test to predict malignant transformation[24].

Studies on allelic imbalances (AI)[25-27] and loss of heterozygozity (LOH)[28] have demonstrated a difference between dysplastic lesions and OLP, with losses at the 3p, 9p and 17p in the former, increasing in number with severity of the dysplasia. Additional LOH at 3p has been detected in OLP by two groups[25,28]. These show that lichen planus can harbour genetic changes.

The aim of this study was to apply image-based DNA ploidy analysis as a predictive test for malignant transformation in a retrospective series of patients with OLP, some of whom had subsequently developed oral squamous cell carcinoma (OSCC). As convincing cases of transformation in OLP are rare, additional clinically suspicious cases were also analysed in an attempt to investigate lesions that might lie in the overlap between OPMD and OLP.

Materials and methods

The study was approved by the Guy's Hospital Research Ethics Committee and the use of material and data by the UK Patient Information Advisory Group (reference PIAG 4-09(f)2003).

Patient selection

Two test groups of patients were selected. The first comprised fourteen patients with a clinico-pathological diagnosis of oral lichen planus, who subsequently developed carcinoma at the same site or in an adjacent part of the oral cavity. All were under long-term follow-up at the Oral Medicine clinic at Guy's Hospital, London, eleven for periods preceding their carcinoma. The clinical diagnosis of lichen planus in these patients had been made on the basis of typical features: bilateral and symmetrical reticular striae or papules affecting primarily the buccal mucosa, with or

without involvement of lateral tongue and gingiva and with or without atrophic ('erosive') areas. Many had had either a rash or history of a typical rash of pruritic papules affecting wrists or shins, with or without additional genital involvement. All patients had at least one biopsy histologically consistent with oral lichen planus and without epithelial dysplasia. Patients that presented with overlapping features suggesting proliferative verrucous leukoplakia (PVL) on presentation were excluded.[23] Some patients developed dysplasia and some had plaques in addition to more typical features of OLP in subsequent biopsies during the evolution of their malignant disease. There are no absolute histological diagnostic criteria for lichen planus and the diagnosis is made partly by exclusion, but all patients met published criteria[35] on presentation and first biopsy, exceeding the stringency applied in other published series[14], as this study required both classical clinical and histological features. Cases were only accepted as cases of malignant transformation in lichen planus after review of clinical records, images if available, and biopsy. Clinical features are shown in table 2.

Unusual lichen planus group: This comprised 42 patients with lichen planus, both clinically and histologically, as for the classical lichen planus, except that unilateral lesions were accepted, together with additional unusual features that might suggest a predisposition to malignant transformation. These included an older age at first diagnosis, a history of smoking, lesions at unusual oral sites (e.g. the floor of the mouth, soft palate), absence of skin lesions, or a prominent erosive component. Patients were excluded if there was any history of potentially causative drugs or contact lichenoid lesions.

Two groups of patients were selected as controls, for whom follow-up data confirmed that they had not developed oral squamous carcinoma subsequent to diagnosis.

Classical lichen planus group: This comprised 68 patients who were diagnosed with oral lichen planus using criteria described by Holmstrup and co-workers[35]. These were patients with mean age of 55.6 ($\pm$ 9.9) years with bilateral and symmetrical white striae in the buccal mucosae and possible concomitant cutaneous lesions, who were non-smokers and were not taking potentially lichenoid reaction-inducing drugs. Histologically, all showed a band-like lymphocytic infiltrate, interface stomatitis with

basal cell apoptosis and basal cell loss, changes in epithelial thickness and keratinisation, but not dysplasia. Cases were excluded if the lichenoid inflammatory infiltrate contained a prominent macrophage or eosinophil component, or there were deep perivascular infiltrates of any cell type.

Methodological control group: This comprised tissue from 80 fibroepithelial polyps from 80 patients who were non-smokers matched by year of diagnosis to the range and year distribution of test samples to allow equivalent follow-up and control for any archival tissue degradation.

Tissue preparation for ploidy analysis

Specimen preparation and ploidy analysis were performed according to Diwakar et al. 2005[36].

Mucosa for analysis was selected on haematoxylin and eosin stained sections. Epithelium was microdissected by scoring the block surface, from which 50μm sections were taken, deparaffinized in xylene, rehydrated in ethanols to PBS, and incubated in 2 mL 0.05% protease type XXIV (Sigma) at 37°C for 90 minutes in a shaking waterbath. The suspension was filtered through a 60-μm nylon mesh (Millipore NY6000010) to separate debris and the nuclei were centrifuged as dispersed monolayers on microscope slides (Cytospin 4; Shandon).

Image-based ploidy analysis

Nuclear monolayers were stained with Feulgen–Schiff and DNA content was measured using a Fairfield ploidy system (Medical Solutions), an automated scanning Zeiss Axioplan II microscope (Zeiss), black and white digital camera (Hamamatsu Photonics, model C4742-95), and a 546 nm green filter giving an estimated resolution of 170 nm per pixel in a 1,024 × 1,024 pixel field.

DNA ploidy classification criteria

Nuclei from lymphocytes, keratinocytes and fibroblasts were classified automatically based on a custom rules file. All classifications were subsequently reviewed by a diagnostic histopathologist (EWO). A lesion was classified as diploid if epithelial nuclei formed one peak at 2c and if the number of nuclei at any 4c peak did not exceed 10% of the total. Any sample with aneuploid peaks (outside diploid index of 1.8–2.2c and 3.6–4.4c) containing more than 10% of the epithelial nuclei or with more than 1% epithelial nuclei with DNA content above 5c was classified as aneuploid. Samples were classified as tetraploid when there were more than10% of the total epithelial nuclei in a 4c peak, but no other abnormality. A minimum of 300 epithelial nuclei were assessed and samples with a diploid peak coefficient of variation of greater than 5% were excluded from the analysis, unless below 6% and grossly multiploid and with a high 5c exceeding rate. These criteria met those recommended by the ESACP consensus report[37].

Outcome and statistical analysis

Outcome for malignant transformation was obtained from the Health and Social Care Information Centre and confirmed against local pathology and clinical records. The FEP lesions had a mean follow-up of 13 years (10 to 16 years), both classical and unusual LP lesions had 14 years (10 to 18) and the group that underwent malignant change 24 years (10 to 38). Malignant transformation probability was calculated using the Kaplan-Meier method and differences between aneuploid and diploid lesions were analysed by log-rank test (SPSS 15.0 software, IBM, USA).

RESULTS

In the series of 14 patients who developed carcinoma, 21 individual oral carcinomas were diagnosed, 4 of which were preceded by an aneuploid lesion (patients 1,2,3 and 7) and 16 by one or more diploid lesions, with a mean transformation time of 18.7 months following the first lesion sampled (fig 1 and 2). Additional clinical information regarding this cohort of patients is displayed in table 2. One patient from the 42 in the unusual lichen planus group underwent subsequent

malignant change (Table 1). All of the unusual LP group lesions and all of the methodological control tissues were diploid.

Kaplan Meier analysis of time to malignant transformation revealed no significant difference between diploid and aneuploid precursor lichen planus lesions (p=0.347).

Three of the patients in the OLP-OSCC group were diagnosed with an OSCC on first presentation to the department (patients 12-14), which was treated surgically and the patients placed on long-term follow-up. The diagnosis of lichen planus previously made in other centres could not be confirmed histologically as material was not available, but at presentation and during follow-up they had or developed further lesions that fitted the criteria for OLP both clinically and histopathologically, without dysplasia orally and with or without skin lesions. One of these patients did not present with further OLP-like lesions within the follow-up period (patient 14), however the margins of the excised tumour without dysplasia had histological features typical of OLP and the clinical presentation was sufficiently typical to accept the diagnosis for the purposes of this study.

From the series of 14 patients, 3 had consecutive biopsies from exactly the same site (patients 5, 7 and 10, Fig 1 and Table 2). The remaining 2 patients, despite having an aneuploid OSCC, had pre-existing diploid lesions at the same site, however, in these latter patients, time to transformation was longer (19 and 64 months) than in the patient with an aneuploid lesion (9 months).

Fifteen biopsy specimens taken subsequent to carcinoma development were analysed and only one produced an aneuploid result (Fig 1).

DISCUSSION

Oral lichen planus was classified as premalignant by the WHO in 1977[38] and its status remains unchanged in the recent WHO classification of OPMD[39]. However, the risk

of OLP and lichenoid lesions transforming to carcinoma remains unclear. Lichen planus is not a specific disease but appears to be a set of common clinical and histopathological features produced by different triggers of epithelial damage. Drugs and topical agents are well enough established causes to be classified separately as lichenoid drug reactions, but mild dysplasia can induce the same pathogenic mechanisms and produce similar or indistinguishable clinical and histological features[13]. Identifying mild degrees of dysplasia histologically in a background of lichenoid immune reponse is extremely difficult, so that accurate differentiation of lichen planus from dysplastic lesions is challenging. In this study we have therefore tested DNA ploidy analysis separately on patients with classical OLP, meeting strict diagnostic criteria, listed above, none of whom developed carcinoma, and on a series of patients who had lesions that strongly resembled OLP but whose clinical or histological features might suggest a risk of transformation. These included lichen planus-like lesions in smokers, plaque-type OLP, those with onset at an age older than typical, lesions at unusual oral sites such as the floor of the mouth and soft palate, absence of rash, unilateral lesions, or a predominant erosive component. Many patients exhibited more than one of these unusual features but only one patient from this group developed OSCC. In defining the experimental groups in this way, we attempt to analyse separately classical lichen planus and conditions that might be mistaken for it either clinically or histologically, recognising that these cannot be well-defined experimental groups.

In identifying the patients in whom OLP apparently progressed to carcinoma, we have done our best to eliminate all other OPMD[39]. All included cases met strict clinical and histological criteria at diagnosis and only half of the suspected cases of transformation in OLP in our institution over this time period were accepted for this study. No dysplasia was accepted at initial diagnosis. Three patients were accepted without a preceding history only on the basis of the same criteria (patients 12-14). Although histological assessment was not possible in these individuals prior to carcinoma, they either developed further lesions of typical OLP (patients 12 and 13) without dysplasia or presented initially with features typical of OLP clinically to and histologically (patient 14).

There have been several previous similar studies on OLP, two of which applied a less senstive Feulgen-stained whole section static cytometric method. Femiano and Scully[40] assessed 25 erosive and 20 reticular lichen planus biopsy samples, finding two erosive lesions to be aneuploid and all reticular specimens to be diploid. However, one of the two aneuploid samples was considered to show mild dysplasia, which would have excluded it from our study. Mattila and coworkers[41] studied 80 samples from 70 patients, all with atrophic/erosive lichen planus, and found 41% of samples to be aneuploid. In this study also, many samples were considered dysplastic and the transformation rate was correspondingly high at 7%. Hosni and co-workers[42] found 13 of 39 lichen planus cases to be aneuploid. Yarom and coworkers[43] used a combination of Giemsa cytometry and an *in situ* hybridisation assay against chromosomes 2, 8 and 9 concluded that 28% of 16 cases showed aneuploidy, though with numerous aneuploid cells found in controls. In all these studies and on the basis of our previously calculated predictive values for malignant transformation[24], these rates of aneuploidy reported in OLP appear too high and are incompatible with the expected transformation rate in OLP, or the known diploid status of normal tissues. The differences in results between these studies and ours arelikely to be accounted for by study populations including both OPMD and OLP, high risk cases and methodological differences. No previous work has attempted to separate typical OLP with confirmed long-term benign outcome from unusual or progressing subgroups.

A further study by Biesterfeld *et al.*[44] detected euploid polyploidisation, or complete multiplication of the DNA content. This state seems rare in oral samples, has no known significance. These ploidy findings are out of consensus and difficult to interpret.

An appraisal of these diverse published results highlights the critical nature of analytic methods and diagnostic criteria, all of which differ from those employed here. In the present study, a ploidy diagnosis was only rendered when the coefficient of variation (CV) of the diploid peak was less than 5%. Only then can conclusions be confidently drawn about DNA ploidy status. In our current analyses using separated nuclei, the CV of the diploid peak is usually less than 3% and close to 1% is often achieved. Many published studies omit this key element and use complex methods to calculate

indices of aneuploidy that have no meaning in a diagnostic context or biologically. Only a significant proportion (1%) of epithelial cells exceeding a DNA content of 5c or defined clonal peaks of 10% of the epithelial nuclei harvested have proven predictive value in OPMD. Different methods produce very different results[45,46] and even using similar methods not all groups confirm the predictive value of DNA ploidy analysis[47]. This study therefore benefits from known predictive values for malignant transformation in a large series of other types of oral lesions using the same method.[24]

A recent study demonstrated that allelic imbalance, assessed by LOH and microsatellite instability (MSI), occurred at higher rates in epithelial dysplasia and SCC than in OLP, which showed similar patterns to benign hyperplasia[25]. Similar findings had also been reported by Zhang et al. in 1997[28] and both studies provide a molecular foundation of small scale genetic changes consistent with our finding that typical OLP lesions can harbour large scale genetic changes.

In the present study, eighty lesions of benign fibroepithelial hyperplasia used as a methodological control were all diploid and follow-up of the patients for 10 years showed that none of the patients developed an oral carcinoma. Test and control were date matched for biopsy to eliminate artefacts from archival biopsy, though analysis is not hampered by aging of blocks[24,48].

No carcinoma developed over 10 years in the 68 patients with classical OLP and all lesions were diploid. One patient from the group of 42 patients with unusual clinical features developed an oral carcinoma though all lesions in this group were also diploid. As these patients with 'unusual' lichen planus deliberately included cases with floor of mouth lesions in smokers and plaque-type lesions, it is perhaps surprising that so few were aneuploid or transformed. These are the very cases we considered likely to cause diagnostic difficulty between OLP and other OPMD clinically. Van Meij and coworkers, in their prospective series[14] reported that all cases of carcinoma that developed were in disease that was either clinically or histologically insufficiently typical for definitive diagnosis as OLP. They termed these lesions 'oral lichenoid lesions'. Some, but not all, of our unusual lesions would meet their criteria for oral lichenoid lesions because we required typical clinical features somewhere in the mouth or on the skin and typical histological features. We

prefer to reserve the term lichenoid reaction for drug-related or topically-induced lichen planus-like lesions, for which it is already widely used. We have also avoided using the term atypical lichen planus to describe our unusual cases. Though they are not typical clinically, the word atypical has histological connotations of dysplasia, which was not present in any of our cases.

Overall, in the group of 14 who developed oral carcinoma in OLP, 21 carcinomas were diagnosed, a significant prevalence of second and third primary carcinomas. Eleven of the patients had a preceding biopsy and aneuploidy or tetraploidy preceded the first carcinoma in 4 patients. In interpreting this proportion, it must be accepted that the numbers are low and that the sites of biopsy may well have been away from the site of development of the carcinoma, so that a single biopsy might have a low chance of predicting cancer even when a degree of field change is present. Overall 4 of the 11 patients (patients 1, 2, 3 and 7) showed ploidy anomalies but no dysplasia in tissue sampled prior to carcinoma. Kaplan Meier analysis revealed no significant difference between diploid and aneuploid precursor lichen planus lesions ($p=0.347$). However, numbers are small and analysis is rendered not significant by the false negative diploid results. The clinical value of an aneuploid or tetraploid result is clearly demonstrated, because all 4 of them identified patients who developed OSCC (patients 1, 2, 3 and 7); there were no false positive results.

It might be assumed that DNA ploidy alterations would be found only in oral mucosa when the subsequent oral cancer is aneuploid. The system used in this study can detect an approximately 1% change in total nuclear DNA content and at this threshold of detection a significant proportion of oral carcinomas appear diploid, presumably harbouring genetic changes below this level. We have shown that there is considerable heterogeneity in oral carcinoma DNA ploidy status and that up to 5 samples may be required to detect it[36]. Our patients under review for lichen planus had carcinomas detected while small and we were therefore unable to sample the carcinomas as extensively as in our previous study. It is also possible that increasing chromosomal instability generates more easily detected ploidy abnormalities in older and larger carcinomas. Nevertheless, 6 of the 14 carcinomas were aneuploid, though this did not correlate with the DNA ploidy status of the preceding specimen(s), when present.

It was not the aim of this study to determine whether or not lichen planus is a potentially malignant disorder. Several factors prevent confident differentiation of OPMD and OLP; overlap in clinical and histological features, the clinical presentation may alter over many years, non-dysplastic lesions may undergo malignant transformation and striae may be associated with leukoplakia ('pumice-type' leukoplakia). It is perhaps not surprising that half of our original institution's cases of malignant transformation in lichen planus were excluded on review, and of those which remained, 10 of 14 had plaque-type lesions at some time in their clinical evolution. This raises the possibility that all such lesions are actually OPMD that mimic OLP, particularly PVL, with which OLP shares many clinical and histological features.

The aim of this study was to determine whether DNA ploidy analysis could be of value in predicting malignant transformation in this difficult area. The selection criteria for carcinoma developing in OLP were stringent, but the results are equally applicable whether these lesions are truly OLP or OPMD indistinguishable from it. We suggest that the positive predictive value of aneuploidy for development of carcinoma in OLP is approximately 36%. Histological assessment would not have predicted transformation in any of these cases because, by definition, cases with dysplasia were excluded from the diagnosis of OLP. While these data are insufficient to suggest that DNA ploidy should be used routinely in such a common condition, DNA ploidy analysis may be useful to assess risk in this difficult area and can detect risk in specimens that would otherwise raise no suspicion.

REFERENCES

1 - Shiohara T, Mizukawa Y, Takahashi R, Kano Y. Pathomechanisms of lichen planus autoimmunity elicited by cross-reactive T cells. Curr Dir Autoimmun 2008;10: 206-226.

2 - Baccaglini L, Thongprasom K, Carrozzo M, Bigby M. Urban legends series: lichen planus. Oral Dis 2013;19:128-143

3 - Pindborg JJ, Bhatt M, Devanath KR, Narayana HR, Ramachandra S. Frequency of oral white lesions among 10,000 individuals in Bangalore, South India. A preliminary report. Indian J Med Sci 1966;20:349-352.

4 - Downer MC, Evans AW, Hughes Hallet CM, Jullien JA, Speight PM, Zakrzewska JM. Evaluation of screening for oral cancer and precancer in a company headquarters. Community Dent Oral Epidemiol 1995;23:84-8.

5 - Axell T, Rundquist L. Oral lichen planus – a demographic study. Community Dent Oral Epidemiol 1987;15:52–6.

6 - Hogewind WF, Van Der Waal I. Prevalence study of oral leukoplakia in a selected population of 1000 patients from The Netherlands. Community Dent Oral Epidemiol 1988 16: 302–5.

7 - Banoczy J, Rigo O. Prevalence study of oral precancerous lesions within a complex screening system in Hungary. Community Dent Oral Epidemiol 1991;19: 265–7.

8 - Albrecht M, Banoczy J, Dinya E, Tamas G Jr. Occurrence of oral leukoplakia and lichen planus in diabetes mellitus. J Oral Pathol Med 1992;21:364–6.

9 - McCartan BE, Healy CM. The reported prevalence of oral lichen planus: a review and critique. J Oral Pathol Med 2008 Sep;37(8):447-53.

10 - Lim K, Moles DR, Downer MC, Speight PM. Opportunistic screening for oral cancer and precancer in general dental practice: results of a demonstration study. Br Dent J 2003; 194: 497-502.

11 - Holmstrup P. The controversy of a premalignant potential of oral lichen planusis over. Oral Surg Oral Med Oral Pathol 1992;73(6):704-6.

12 - Eisenberg E, Krutchkoff DJ. Lichenoid lesions of oral mucosa.Diagnosticcriteria and their importance in the alleged relationship to oral cancer. Oral Surg Oral Med Oral Pathol 1992;73(6):699-704.

13 - van der Waal I. Oral potentially malignant disorders: is malignant transformation predictable and preventable? Med Oral Patol Oral Cir Bucal 2014;19(4):e386-90.

14 - Meij, EH van der, Mast H, Waal, I van der. The possible premalignant character of oral lichen planus and oral lichenoid lesions: a prospective five-year follow-up study of 192 patients. Oral Oncol 2007;43:742-8.

15 - Bombeccari GP, Guzzi G, Tettamanti M, Gianni AD, Baj A, Pallotti F. Oral lichen planus and malignant transformation: a longitudinal cohort study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:328–34.

16 - Meij, EH van der & Waal, I van der. Lack of clinicopathologic correlation in the diagnosis of oral lichen planus based on the presently available diagnostic criteria and suggestions for modification. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endod 2003;32:507-12.

17 - Krutchkoff DJ, Eisenberg E. Lichenoid dysplasia: a distinct histopathologic entity. Oral Surg Oral Med Oral Pathol 1985;60(3):308-15.

18 - Smith J, Rattay T, McConkey C, Helliwell T, Mehanna H. Biomarkers in dysplasia of the oral cavity: a systematic review. Oral Oncol 2009;45(8):647-53.

19 - Pitiyage G, Tilakaratne WM, Tavassoli M, Warnakulasuriya S. Molecular markers in oral epithelial dysplasia: review. J Oral Pathol Med 2009;38(10):737-52.

20 - Abdel-Salam M, Mayall BH, Chew K, Silverman S Jr, Greenspan JS. Prediction of malignant transformation in oral epithelial lesions by image cytometry. Cancer 1988;62(9):1981-7.

21 - Maraki D, Becker J, Boecking A. Cytologic and DNA-cytometric very early diagnosis of oral cancer. J Oral Pathol Med 2004;33(7):398-404.

22 - Torres-Rendon A, Stewart R, Craig GT, Wells M, Speight PM. DNA ploidy analysis by image cytometry helps to identify oral epithelial dysplasias with a high risk of malignant progression. Oral Oncol 2009;45(6):468-73.

23 - Klanrit P, Sperandio M, Brown AL, Shirlaw PJ, Challacombe SJ, Morgan PR et al. DNA ploidy in proliferative verrucous leukoplakia. Oral Oncol 2007;43(3):310-6.

24 - Sperandio M, Brown AL, Lock C, Morgan PR, Coupland VH, Madden PB, et al. Predictive value of dysplasia grading and DNA ploidy in malignant transformation of oral potentially malignant disorders. Cancer Prev Res (Phila) 2013;6(8):822-31.

25 - Accurso BT, Warner BM, Knobloch TJ, Weghorst CM, Shumway BS, Allen CM, Kalmar JR. Allelic imbalance in oral lichen planus and assessment of its classification as a premalignant condition. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011 Sep;112(3):359-66.

26 - Partridge M, Pateromichelakis S, Phillips E, Emilion GG, A'Hern RP, Langdon JD. A case-control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization. Cancer Res 2000; 60: 3893–8.

27 - Partridge M, Emilion G, Pateromichelakis S, A'Hern R, Phillips E, Lang- don J. Allelic imbalance at chromosomal loci implicated in the pathogenesis of oral

precancer, cumulative loss and its relationship with progression to cancer. Oral Oncol 1998; 34: 77–83.

28 - Zhang L, Michelsen C, Cheng X, Zeng T, Priddy R, Rosin MP. Molecular analysis of oral lichen planus. A premalignant lesion? Am J Pathol 1997 Aug;151(2):323-7.

29 - Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. J Oral Pathol Med 2008;37(3):127-33.

30 - Warnakulasuriya S, Kovacevic T, Madden P, Coupland VH, Sperandio M, Odell E, et al. Factors predicting malignant transformation in oral potentially malignant disorders among patients accrued over a 10-year period in South East England. J Oral Pathol Med 2011;40(9):677-83.

31 - Fleskens S, Slootweg P. Grading systems in head and neck dysplasia: their prognostic value, weaknesses and utility. Head Neck Oncol 2009;11;1:11.

32 - Hogewind WF, van der Kwast WA, van der Waal I. Oral leukoplakia, with emphasis on malignant transformation. A follow-up study of 46 patients. J Craniomaxillofac Surg 1989;17(3):128-33.

33 - Holmstrup P, Vedtofte P, Reibel J, Stoltze K. Long-term treatment outcome of oral premalignant lesions. Oral Oncol 2006;42(5):461-74.

34 - Dost F, Lê Cao K, Ford PJ, Ades C, Farah CS. Malignant transformation of oral epithelial dysplasia: a real-world evaluation of histopathologic grading. Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117(3):343-52.

35 - Holmstrup P, Thorn JJ, Rindum J, Pindborg JJ. Malignant development of lichen planus-affected oral mucosa. J Oral Pathol 1988;17(5):219-25.

36 - Diwakar N, Sperandio M, Sherriff M, Brown A, Odell EW. Heterogeneity, histological features and DNA ploidy in oral carcinoma by image-based analysis. Oral Oncol 2005;41(4):416-22.

37 - Haroske G, Baak JP, Danielsen H, Giroud F, Gschwendtner A, Oberholzer M, et al. Fourth updated ESACP consensus report on diagnostic DNA image cytometry. Anal Cell Pathol 2001;23:89–95.

38 - World Health Organization Collaborating Centre for oral precancerous lesions. Definition of leukoplakia and related lesions an aid to studies on oral precancer. Oral Surg Oral Med Oral Pathol 1978; 46; 518-39.

39 - Warnakulasuriya S, Johnson N, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007; 36; 575-580.

40 - Femiano F, Scully C. DNA cytometry of oral leukoplakia and oral lichen planus. Med Oral Patol Oral Cir Bucal 2005;10 Suppl 1:E9-14.

41 - Mattila R, Alanen K, Syrjänen S. DNA content as a prognostic marker of oral lichen planus with a risk of cancer development. Anal Quant Cytol Histol 2004;26(5):278-84.

42 - Hosni ES, Yurgel LS, Silva VD. DNA ploidy in oral lichen planus, determined by image cytometry. J Oral Pathol Med 2010 Mar;39(3):206-11.

43 - Yarom N, Shani T, Amariglio N, Taicher S, Kaplan I, Vered M, Rechavi G, Trakhtenbrot L, Hirshberg A. Chromosomal numerical aberrations in oral lichen planus. J Dent Res 2009 May;88(5):427-32.

44 - Biesterfeld S, Füzesi L, Härle F, Böcking A. DNA-cytometric detection of euploid polyploidization in oral lichen ruber planus. Anal Quant Cytol Histol 1991;13(1):7-10.

45 - Brouns EREA, Bloemena E, Belien JAM, Broeckaert MAM, Aartman IHA, Waal I van der. DNA ploidy measurement in oral leukoplakia: different results between flow and image cytometry. Oral Oncol 2012;48(7):636-640.

46 - Bradley G, Odell EW, Raphael S, Ho J, Benchimol S and Kamel-Reid S. 2010. Abnormal DNA content in oral epithelial dysplasia is associated with increased risk of progression to carcinoma. Br J Cancer 103:1432-42.

47 - Bremmer JF, Brakenhoff RH, Broeckaert MAM, Beliën JAM, Leemans CR, Bloemena E, et al. Prognostic value of DNA ploidy status in patients with oral leukoplakia. Oral Oncol 2011;47(10):956-960.

48 - Stenersen TC, Danielsen H, Farrants G, Reith A. Caveats in the use of archival cell material for DNA ploidy analysis by image cytometry. Anal Cell Pathol 1994;7(3):217-33.

Figure 1. DNA ploidy status of biopsies and carcinomas in the patients who developed carcinoma in lichen planus.

Left, patient number. Each row of symbols shows sequential samples from each patient showing the DNA ploidy status of each biopsy on a non-linear time line with first carcinoma aligned on the vertical line. Circles, biopsies without invasion (including lichen planus specimens); squares, OSCC. Shading shows ploidy status: unfilled, diploid; grey, tetraploid; black, aneuploid. Numbers between symbols are months between biopsies. Symbols joined by lines are separated by short time periods and analysed together.

Figure 2. Example ploidy histograms as stacked bar charts, X axis integrated optical density calibrated as ploidy (2c = diploid), Y axis number of nuclei. Green, epithelial nuclei; blue, control lymphocytes. Left, sample from lichen planus case showing a diploid peak only, CV 1.65, n=1262 epithelial nuclei. Middle, lichen planus followed by carcinoma, sample from patient 3 biopsy 2 showing an aneuploid peridiploid peak at diploid index 1.1 and a small corresponding 4c peak, CV diploid peak 1.81, n= 1533. Right, histological appearances of the aneuploid sample showing parakeratosis, basement membrane thickening and loss of basal cells with lymphocyte infiltration of the basal and suprabasal cell layers associated with apoptosis and colloid body formation. There is an underlying lymphocytic infiltrate, though this is not band-like. No dysplasia is present and the appearances are consistent with lichen planus.

Table 1. Numbers of OLP samples in each group, their DNA ploidy result and malignant transformation.

Table II. Clinical features of the 14 patients with OLP who underwent malignant transformation.

Table 1. Numbers of OLP samples in each group, their DNA ploidy result and malignant transformation.

Tissue	N	Diploid	Tetraploid	Aneuploid	Transformed (%)
Unusual OLP	42	42	0	0	1 (2.4)
Classical OLP	68	68	0	0	0 (0)
Control FEP	80	80	0	0	0 (0)
TOTAL	190	190	0	0	1 (n/a)

OLP = lichen planus, FEP = fibro-epithelial polyps.

Table II. Clinical features of the 14 patients with OLP who underwent malignant transformation.

Patient	Gender	Age at 1st LP biopsy	LP Site(s) biopsy	Features on presentation	Features developed subsequently	SCC Site
1	F	29	BM, G, P, T	S, E, P		BM
2	F	51	BM, G, HP, SP	E, P, DG		G(ur)
3	F	48	BM(bil), FOM, G	S, E, P		G(lr)
4	M	35	BM, T	S, P		T(lb)
5	F	53	T	P	S	T(lb)*
6	F	54	BM	S	E	BM(r)
7	M	74	T	P	S, E	T(lb)*
8	M	50	BM	S, E		BM/G(l)
9	F	55	BM(bil), G(all q)	S	DG, E	G (llq)
10	F	59	T	S, P		T*
11	F	62	BM(bil)	S, P	E	G(llq)
12	F	49	BM(bil)	S, E	P	BM(l)
13	F	74	LM	S		BM
14	F	59	BM(bil), T	S, E, P		T(lb)

Sites: BM (buccal mucosa), G (gingiva), P (palate), T (tongue), HP (hard palate), SP (soft palate), FOM (floor of the mouth), LM (labial mucosa).
Subsites: lb (lateral border), r (right side), l (left side),bil (bilateral), q (quadrant), ur (upper right), lr (lower right), llq (lower left quadrant).
Features: S (striae), E (erythema), P (plaque), DG (desquamative gingivitis)
* indicates SCC developing at the same site as the original LP biopsy.