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Chromosome instability predicts the progression of premalignant oral lesions

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SUMMARY

Objectives: One of the main problems in reducing the incidence of oral squamous cell carcinoma (OSCC) is the inability to appropriately deal with leukoplakia. Accurately identifying lesions which will progress to malignancy is currently not possible. The present study aims to establish the value of chromosome instability (CI) detection by DNA image cytometry and FISH analysis for prognosis and monitoring of oral leukoplakia.

Materials and methods: For this purpose, we included from our archives 102 oral leukoplakia cases, which had been diagnosed between 1991 and 2008. Patient follow-up data were collected and the histopathological diagnosis was revised. CI assessment was carried out on paraffin-embedded tissue sections using both DNA image cytometry (ICM) and dual target FISH for chromosomes 1 and 7.

Results: 16 of 102 Patients developed carcinoma in situ or OSCC. Both detection methods were found to yield prognostic information independent of the histopathological diagnosis. CI was a strong individual marker of progression, with hazard ratios (HRs) of 7.2 and 6.8 for ICM and FISH respectively. Moreover, this approach seems suitable for monitoring lesions over time (especially ICM). Combining histopathology and CI enables subdivision of patients into three risk groups, with different probabilities of malignant progression.

Conclusion: CI detection seems a reliable method for risk assessment of oral premalignancies and its application may contribute to a better risk-counselling and appropriate treatment regimen or watchfull-waiting approach of patients.

Introduction

Oral cancer is the sixth most common malignancy worldwide, with a yearly incidence of about 274.000 cases [1]. Most oral tumours are squamous cell carcinomas (SCCs) with smoking and alcohol consumption as major risk factors [1,2]. In contrast to cancers of the breast, colon, prostate and melanoma that are showing a significantly better prognosis due to improvements in early detection and therapy [3], the survival rate of patients with oral squamous cell carcinomas (OSCCs) has not increased substantially over the last decades [2,4]. The oral cavity and oropharynx are easily accessible for visualization, which may facilitate early diagnosis of (pre)malignant lesions. Nevertheless, in spite of advances in surgical and other treatment modalities, the 5-year survival rate of OSCC remains only approximately 50% [2,3]. This lack of progress can partially be explained by our inability to adequately recognize early developed OSCC as well as precursor lesions at risk for progression [3].

Oral leukoplakia and, to a lesser degree, erythroplakia are relatively common lesions of the oral mucosa, some of which are at risk for malignant transformation [5]. In particular, the borders of the tongue and the floor of the mouth have been mentioned as high-risk sites [6]. Whereas oral erythroplakia show malignant transformation in almost all cases, oral leukoplakia eventually progress to malignancy in an estimated 1.1–17.5% [7]. Therefore, especially discrimination between potentially malignant leukoplakias and non-harmful lesions is of key importance.

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Unfortunately, current histopathological examination of precursor lesions (i.e. dysplastic and non-dysplastic) is insufficiently accurate to predict their malignant potential [8]. In addition, histopathological classification is hampered by observer variability [9,10]. and sampling variability [11]. Therefore, more effective methods for the assessment of the malignant potential of oral leukoplakia are urgently needed [12]. It is widely believed that instability of the genome is the driving force behind the development of the majority of human epithelial cancers [13]. CI may be detected by different molecular biological approaches. DNA content analysis using image cytometry (ICM) or flow cytometry (FCM) are frequently used techniques [4,14–17]. Three recently published retrospective studies have shown that ICM is able to identify patients with dysplasias that are more likely to progress to OSCC [14,16,17]. FCM displays an increasing degree of aneuploidy in the spectrum of lesions ranging from healthy oral mucosa, non-dysplastic and dysplastic leukoplakia to OSCC [15]. Alternatively, copy number alterations of individual chromosome loci can be studied using fluorescence in situ hybridization (FISH) analysis [18–21]. Several studies on FISH for chromosomes 1 and 7 revealed that numerical chromosome aberrations have a predictive value in the progression of oral precursor lesions to SCC [18,19,22].

The aim of the present study was to establish the value of CI detection by means of both ICM and FISH in comparison with routine histopathological assessment for the risk assessment of oral precursor lesions in a series of 102 leukoplakia. In addition, we evaluated both essays for their reliability to monitor possible progression of premalignant lesions over time.

Materials and methods

Patient selection

This retrospective study included patients who were admitted to the department of Oral and Maxillofacial Surgery at the Radboud University Nijmegen Medical Centre (RUNMC), The Netherlands, between 1991 and 2008 for evaluation of suspicious lesions of the oral mucosa. The minimal follow-up time was 6 months. Patients with oral hyperplastic or dysplastic lesions were identified using the Dutch Pathology diagnosis and registration system (PALGA). Patients who had been previously treated for malignancy as well as patients with a histologically confirmed carcinoma in situ at first biopsy were excluded. Clinical data including age, etiological factors, localization, type of treatment (surgery, laser ablation or expectative policy) were obtained from the patients' medical charts. Only patients with a complete data-set were included, resulting in 102 specimens (of 102 patients). Data collection was performed in accordance with local ethical guidelines.

ICM and FISH analyses were performed on biopsy specimens obtained at the time of the first diagnosis of leukoplakia and on specimens from eventually developed carcinoma (carcinoma in situ or invasive carcinoma). The time between consecutive lesions was set at a minimum of 6 months in order to exclude synchronous lesions. To assess the value of ICM and FISH for the monitoring of lesions over time, subsequent premalignant lesions and recurrent malignancies were analysed.

For each case of leukoplakia and SCC first a 4 μm thick slide was cut from a paraffin-embedded specimen for Haemotoxylin-eosin (HE) analysis followed by three 50 μm and two 4 μm sections for ICM and FISH respectively and ending again with one 4 μm slide for HE analysis. The first and last slides of the initial leukoplakia were haematoxylin-eosin stained and revised by an experienced pathologist (PJS). In this manner it could be assured that both ICM and FISH analysis were performed on representative areas, to enhance the accuracy of the test.

DNA ploidy measurement of isolated nuclei from tissue sections

Two to four 50 μm thick paraffin-embedded sections were used to isolate cell nuclei according to well-established procedures [10]. A thickness of 50 μm was shown to minimize the number of artefacts in DNA histograms [23]. Sections were deparaffinised and rehydrated as follows: xylene (three times 15 min), 100% ethanol (twice), 96% ethanol (twice), 70% ethanol, 40% ethanol, distilled water and phosphate-buffered saline (PBS) at least 10 min in each solution. The tissue sections were transferred into a centrifuge tube and incubated with 0.5% pepsin (Sigma Aldrich, St Louis, MO, USA) in PBS (pH 1.50) at 37 °C, for 60 min. After enzymatic digestion, 4 ml of ice-cold PBS was added to stop the reaction. Next the tissue suspension was filtered through a CellTrics filter with 50 μm pore size to separate nuclei from tissue debris. After centrifugation (1000 rpm, 10 min), 2 ml PBS was added and the amount of nuclei was counted with a cell-counting device (Z1 Coulter Particle Counter®, Beckman Coulter Inc., Woerden, The Netherlands). The cell suspension was diluted until the right concentration, (20,000 cells) was reached, to form a monolayer in a cytospin-centrifuge (10 min 700 rpm), air dried, and fixed in Böhm fixative for 1 h, hence a mixture of 85% methanol absolute, 10% formaldehyde (37%), and 5% acetic acid (96%). Slides were air dried after twice rinsing in methanol absolute.

Feulgen staining

First the cell preparations were hydrolysed in 5 M HCl under controlled temperature at 25 °C for 1 h, after which the process was stopped using distilled water. Thereupon the isolated nuclei were stained by the Schiff method [10] (Merck®, Darmstadt, Germany) for 1 h at room temperature. Next the slides were rinsed in streaming tap water for 20 min, dehydrated in increasing concentrations of ethanol, xylol and mounted in Permount® (Thermo Fisher Scientific, Landsmeer, The Netherlands).

Measurement of DNA content

All measurements were performed using the Leica QPath Image Cytometry Workstation (Leica Microsystems GmbH, Wetzlar, Germany) according to an established protocol [10]. Slides of stained cell nuclei were analysed using a microscope equipped with a 550-nm green filter and a computer controlled stage. Each monolayer contained at least 1500 sampled nuclei. The QPath software measures the Feulgen integrated optical density (IOD) of individual nuclei while avoiding the measurement of overlapping, folded, or clumped cells. ICM analysis of all histological specimens was performed in a blinded fashion at the RUNMC. DNA ploidy histograms were visually classified independently by two experienced observers (JvdL; IOH). In all cases of discrepancy, a consensus classification was reached by the same two observers.

In the present study, a diploid histogram is defined by a single dominant 2c peak (representing G_0/G_1 phase cells) and possibly a 4c peak (G_2 phase cells) not exceeding 10% of the total number of nuclei (Fig. 1A). If the number of nuclei with a DNA content >5c exceeded 1% of the total number of cells, the histogram was classified as aneuploid. Also, a histogram was classified as aneuploid if there was a clear and distinct peak outside the 2c/4c regions (Fig. 1B). A lesion was defined as tetraploid if the number of 4c nuclei exceeded 10% of the total number of epithelial cells and a 8c peak was observed. The measurements were performed according to previous published guidelines [24]. Because in some cases the coefficient of variation (CV: standard deviation of the 2c peak divided by the mean, given in percent) slightly exceeded the advised threshold of 5%, CVs up to 6% were allowed in the present study.

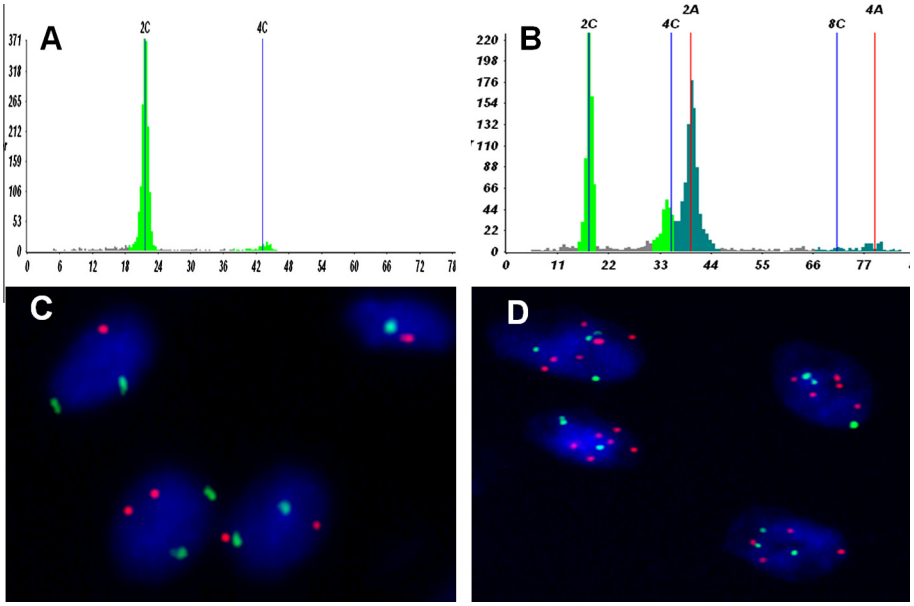

Figure 1. A, B Examples of ICM ploidy histograms **A**. diploid pattern, **B**. typical aneuploid pattern. **C, D** Representative examples of FISH results using centromere probes specific for chromosome 1 (green) and 7 (red) in cell nuclei (blue) of head and neck oral mucosa epithelium. **C.** Nuclei showing disomy for both chromosomes. **D.** Nuclei showing an imbalance between the copy number of chromosomes 1 (maximal 4 copies) and 7 (maximal 6 copies).

Double-target fluorescence in situ hybridisation (FISH) on tissue specimens

FISH was performed at the University of Maastricht Medical Centre, Department of Molecular Cell Biology. The procedure was performed as described previously [10,25]. Briefly, 4 μm thick paraffin-embedded tissue specimens were first deparaffinised and pretreated with 85% formic acid/0.3% H_2O_2 at room temperature 1 M sodium thiocyanate (NaSCN) at 80 °C and 0.4 g pepsin (800–1200 U/mg protein porcine stomach mucosa (Sigma Aldrich, St Louis, MO, USA) per ml 0.02 NHCl at 37 °C. Slides were post-fixed with 1% formaldehyde in PBS and dehydrated with an ascending series of ethanol. The probes for chromosome 1 and 7 were prepared by labelling with biotin- and digoxigenin-dUTPs and dissolved at a concentration of 1 ng/μl in 60% formamide, 2× standard saline citrate (SSC, pH 7.0), 10% dextran sulphate, and 50 times excess of carrier DNA (salmon testis DNA; Sigma Chemical Co., St. Louis, MO). After denaturation of probe and tissue at 80 °C for 5 min and overnight hybridization the tissue specimens were washed stringently in 2× SSC containing 0.05% Tween-20 (Acros Organics, Geel, Belgium) two times for 5 min at 42 °C, followed by 0.1 × SSC two times for 5 min at 60 °C and 4 × SSC/0.05% Tween-20 at room temperature.

Detection of the hybridised probes was established with the following antibody combinations diluted in 4 × SSC containing 5% non-fat dry milk powder: FITC-labeled avidin (Vector, 1:100)/anti-digoxigenin monoclonal antibody (1:200, Mannheim); Biotinylated Goat Anti-Avidin (Vector, 1:100)/rabbit anti mouse TRITC (Vector, 1:1000) and FITC-labeled avidin (1:100)/swine anti rabbit TRITC (Vector, 1:100). Following the final wash and dehydration, slides were mounted in Tris glycerol, containing 2% DACO Antifade (Glostrup, Denmark) and 0.5 μg/ml of the nuclear DNA counterstain DAPI. An example of FISH results is shown in Fig. 1C and D. Evaluation of the FISH results was carried out as described in our previous studies [10,22,25].

Data analysis

Clinical data, histopathological characteristics and ICM and FISH scores of all lesions were statistically analysed using SPSS version 17.0.1 software (SPSS Inc., Chicago, USA). The progression into carcinoma was analysed using uni- and multivariate Cox regression analyses and hazard ratios (HRs) including its 95% confidence intervals (CIs) and associated *p*-values were calculated. Cumulative progression towards malignancy analysis was performed by means of the Kaplan Meier method. In all analyses the *p*-value for significance was set at 0.05. To assess the degree of agreement between ICM and FISH κ-statistics were calculated.

Results

Patient characteristics and outcome

Of the 102 patients diagnosed with leukoplakia, 48 (47%) were female and 54 (53%) were male (Table 1). The duration of follow-up after the first biopsy ranged from 6 to 246 months (median 91.5). Malignant progression occurred in 16 patients (5 developed

Table 1
Patient characteristics based upon primary premalignancies.

Patient data		Malignant Transformation	
		No	**Yes**
Number of patients		86	16
Age	Mean (years)	51.9	57.8
	CI (95%)	(49.6–54.2)	(50.9–64.7)
Sex	Female	41	7
	Male	45	9
Smoking	Yes	54	9
	No	32	5
	In history	9	1
	Data NA	2	1
Alcohol	Yes	39	7
	No	40	8
	Data NA	4	1
Follow-up	Median (months)	98.0	27.0
	CI (95%)	(84.8–104.6)	(23.7–70.8)

NA = not available.

carcinoma in situ and 11 invasive carcinoma). The median follow-up time of these patients was 27.0 months (range: 7–151). Progressive lesions were located on the floor of mouth ($n = 2$), tongue ($n = 10$), buccal mucosa ($n = 3$) and inferior alveolar rim ($n = 1$). Carcinomas developed at the same location as the preceding leukoplakias ($n = 13$), or within a range of 2 cm of the initial lesion ($n = 3$) (Warren and Gates criteria) [26].

Clinical parameters

Table 3 gives an overview of the HRs of different clinical parameters. Location was associated with risk of malignant development: leukoplakia of the floor of mouth or tongue had a significantly higher risk of malignant progression (HR = 3.2; $p = 0.047$). Older patients were slightly more susceptible to malignant transformation with a HR of 1.1 ($p = 0.025$). No correlation was found between the type of treatment and developed SCC (data not shown).

Histopathology

An overview of the histological diagnosis for each case of leukoplakia related to malignant progression can be found in Table 2 (first two columns). Because of the low number of severe dysplasias ($n = 3$), in the present study these lesions were pooled with moderate dysplasias for further analyses. This is in accordance with the review by Warnakulasuriya et al. [27]. In univariate Cox regression, histopathological diagnosis was associated with malignant transformation with a hazard ration (HR) of 6.3 (CI: 2.3–17.3; $p < 0.001$; Table 3) comparing the combination of hyperplasia and mild dysplasia with moderate and severe dysplasia. Fig. 2 shows the corresponding survival curves.

Chromosome instability

As tetraploid lesions ($n = 3$ for both ICM and FISH) harbour an abnormal DNA content [28–30], these lesions were included in the aneuploid group. ICM analysis revealed 23 aneuploid vs 79 diploid lesions (23%) and FISH showed 17 aneuploid vs 85 diploid lesions (16%). Comparison of FISH and ICM revealed an identical outcome in 90 out of 102 leukoplakia (Table 4). The kappa statistic for this comparison was 0.63, which indicates a substantial agreement between both methods. (CI: 0.44–0.82). The percentage of aneuploidy increased with advancing histopathological classification. Of the aneuploid lesions detected by respectively ICM and FISH, 10 of 23 (43.5%) and 8 of 17 (47.1%) showed malignant progression. In contrast, only 6 out of 79 (7.6%) and 8 of 85 (9.4%) of diploid leukoplakia showed malignant progression for ICM and FISH, respectively (Table 2).

Univariate Cox regression analysis revealed an increased risk of malignant progression for aneuploid lesions (both ICM (HR = 7.2; $p < 0.001$) and FISH (HR = 6.8; $p < 0.001$); Table 3). This is also reflected in the progression-free survival curves based on CI detection, showing a significantly shorter survival for aneuploid lesions (Figs. 3 and 4). Including both FISH and ICM in multivariate Cox regression analysis showed decrease of the HR for both techniques (ICM: HR = 4.2; $p = 0.052$ respectively FISH: HR = 2.3; $p = 0.240$; Table 3) for ICM and FISH, respectively), indicating that both techniques yield comparable information.

Combining histopathology and CI

In multivariate Cox regression analysis, ICM adjusted for histopathology showed a HR of 5.4 (CI: 1.82–15.8; $p = 0.002$; Table 3). In this analysis histopathology and ICM appeared to be rather independent, complementary factors. Therefore, multivariate analysis was also performed by comparing 4 combined classes i.e.: low-grade diploid, high-grade diploid, low-grade aneuploid and high-grade aneuploid lesions (high grade being defined as moderate or severe dysplasia, low grade encompassing the other histopathologic categories). Taking diploid low grade lesions as the reference category, the HR of aneuploid high-grade lesions was found to be as high as 22.0 (CI: 5.78–83.45); $p < 0.001$), with lesions being either high-grade diploid or low-grade aneuploid showing HR of 4.3 (CI: 0.76–23.92; $p = 0.101$) and 5.5 (CI: 1.37–21.96; $p = 0.016$), respectively (Table 5). Because the latter two groups showed comparable HRs they were combined in progression-free survival analysis (Fig. 5).

FISH adjusted for histopathology showed a HR of 4.4 (CI: 1.5–13.1; $p < 0.01$; Table 3). Combining histopathology and FISH in multivariate Cox regression in a manner comparable to that described above for ICM and histopathology, similar results were obtained (Table 5). Aneuploid high-grade lesions show a HR of 16.3 (CI: 3.90–68.18; $p < 0.001$) compared to low grade diploid lesions, with lesions being either high-grade diploid or low-grade aneuploidy showing HR of 9.0 (CI: 2.21–36.9; $p = 0.002$) and 10.1 (CI: 2.52–40.4; $p = 0.001$). When the latter two groups were combined a HR of 9.5 (CI: 2.85–32.00; $p < 0.001$) was found in progression-free survival analysis as compared to low grade diploid lesions, but was not significantly different from aneuploid high-grade lesions (HR 1.71 with CI: 0.49–6.0; $p = 0.40$).

Monitoring

ICM revealed that almost all aneuploid malignancies resulted from aneuploid precursor lesions (9 out of 10) and almost all diploid carcinoma had a diploid precursor lesion (5 out of 6; FISH showed more variability: 4/8 respectively 5/8). Interestingly, two aneuploid leukoplakias developed after 9.3 and 12.6 years into an aneuploid SCC (based on ICM).

Also analysis of intermediate precursor lesions and recurrent SCC was performed (Tables 6 and 7). From seven initial premalignancies showing progression to SCC, subsequent biopsies were available. Leukoplakia and subsequent malignancies were considered to be related if they were found at the same mucosal location.

Six of seven cases (ICM-based) showed that the ploidy status of the successive lesions (both premalignant lesions and SCC) was identical to the status of the initial lesion. Only one initial aneuploid leukoplakia was followed by both a diploid leukoplakia and

Table 2
Fraction of hyperplastic and dysplastic lesions transformed into malignancies.

Histopathology	Number of SCC after leukoplakia	Number of SCC after diploid leukoplakia (ICM)	Number of SCC after aneuploid leukoplakia (ICM)	Number of SCC after diploid leukoplakia (FISH)	Number of SCC after aneuploid leukoplakia (FISH)
All	16/102 (15.7%)	6/79 (7.6%)	10/23 (43.5%)	8/85 (9.4%)	8/17 (47.1%)
HP	5/66 (7.6%)	4/59 (6.8%)	1/7 (14.3%)	4/63 (6.3%)	1/3 (33.3%)
D+	3/16 (18.8%)	0/11 (0.0%)	3/5 (60.0%)	0/11 (0.0%)	3/5 (60.0%)
D++	7/17 (41.2%)	2/9 (22.2%)	5/8 (62.5%)	3/10 (30.0%)	4/7 (57.1%)
D+++	1/3 (33.3%)	0/0 (0.0%)	1/3 (33.3%)	1/1 (100.0%)	0/2 (0.0%)

HP = hyperplastic; D + = mild dysplasia; D++ = moderate dysplasia; D+++ = severe dysplasia.

Table 3
Progression-free period and different assays as well as clinical parameters determined by uni- and multivariate Cox regression analyses. Floor of mouth and tongue were considered to be high-risk locations, all other sites low-grade.

Variable	Univariate Cox regression analysis		Multivariate Cox regression analysis		
	Hazard ratio	*p*-Value	Hazard ratio Corrected for:		
			Histopath.	ICM	FISH
Histopathology	6.3 (2.32–17.25)	<0.001	–	4.1 (1.42–11.80) ($p = 0.009$)	3.9 (1.31–11.50) ($p = 0.014$)
DNA image cytometry	7.2 (2.61–20.03)	<0.001	5.4 (1.82–15.77) ($p = 0.002$)	–	4.2 (0.99–17.59) ($p = 0.052$
FISH	6.8 (2.53–18.30)	<0.001	4.4 (1.50–13.08) ($p = 0.007$)	2.3 (0.57–9.27) ($p = 0.24$)	–
Age	1.1 (1.01–1.10)	0.025			
Sex	1.2 (0.42–3.11)	0.79			
Location	3.2 (1.02–9.82)	0.047			
Treatment	0.9 (0.29–2.93)	0.88			
Alcohol	1.1 (0.98–1.23)	0.098			
Tobacco	0.9 (0.32–2.73)	0.74			
Pack years	1.0 (0.94–1.02)	0.29			

CI = 95% confidence interval.

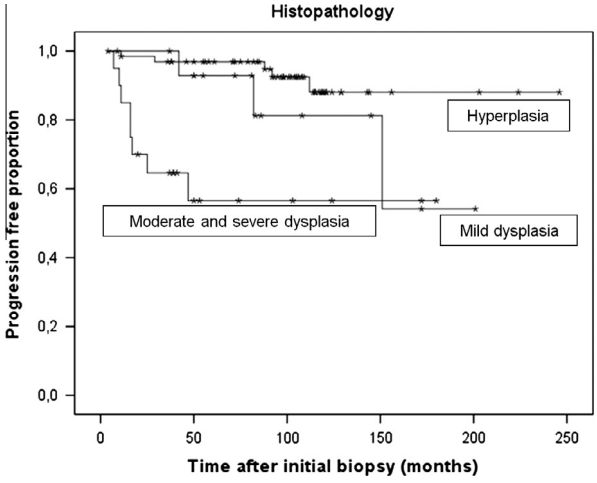

Figure 2. Outcome of patients with leukoplakia of the oral cavity, based upon histopathology. Comparing the combination of hyperplasia and mild dysplasia with moderate and severe dysplasia results in HR = 6.3 (CI: 2.32–17.25; $p < 0.001$).

a diploid SCC (patient 1; Table 6). Interestingly, three initially aneuploid lesions without malignant progression for which subsequent leukoplakia specimens were available, showed transition from aneuploid to diploid lesions (ICM-based) (Table 7). Additionally, 6 (ICM-based) diploid initial leukoplakia without malignant progression were followed by diploid leukoplakia on the same location (FISH: 4 diploid; 2 not available). (data not shown). Five patients treated for SCC developed new leukoplakia at the same location (Table 6).

Table 4
Correlation-table ICM and FISH.

		FISH		
		Diploid	Aneuploid	Total
ICM	Diploid	76	3	79
	Aneuploid	9	14	23
	Total	85	17	102

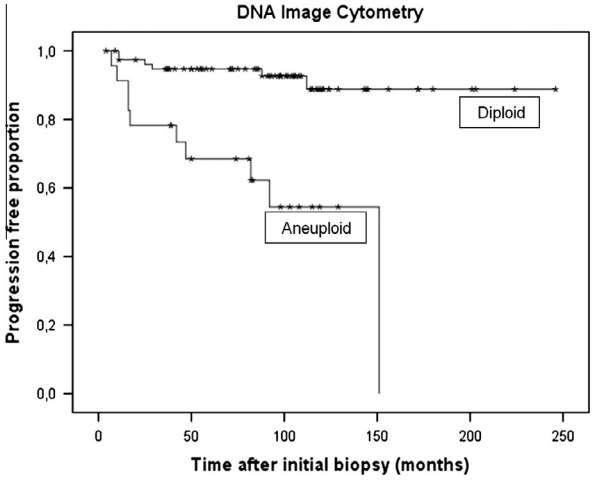

Figure 3. Kaplan–Meier curve of the progression free proportion of based upon ICM. HR = 7.2 (CI: 2.61–20.03; $p < 0.001$).

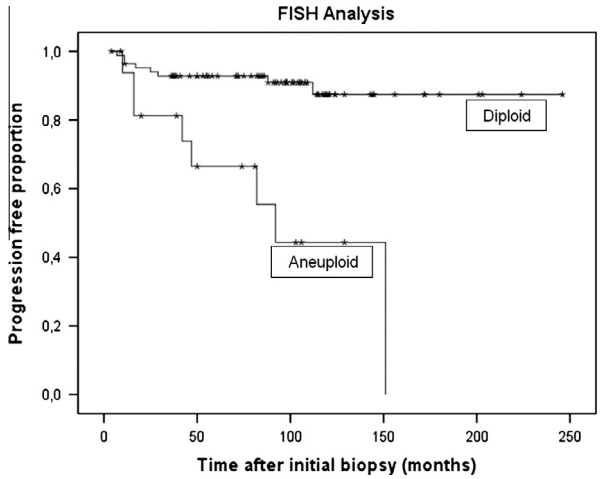

Figure 4. Kaplan–Meier curve of the progression free proportion of based upon FISH. HR = 6.8 (CI: 2.53–18.30; $p < 0.001$).

Table 5
Level of association of ICM/FISH and different histopathological classes with progression free survival.

DNA image cytometry	Low-grade (HP and D+)		High-grade (D++ and D+++)	
Histopathology				
Diploid	Reference	$N = 70$	HR: 4.3 (CI: 0.76–23.92) ($p = 0.101$)	$N = 9$
Aneuploid	HR: 5.5 (CI: 1.37–21.96) ($p = 0.016$)	$N = 12$	HR: 22.0 (CI: 5.78–83.45) ($p < 0.001$)	$N = 11$
FISH analysis				
Diploid	Reference	$N = 74$	HR: 9.04 (CI: 2.21–36.9) ($p = 0.002$)	$N = 11$
Aneuploid	HR: 10.1 (CI: 2.52–40.4) ($p = 0.001$)	$N = 8$	HR: 16.3 (CI: 3.90–68.18) ($p < 0.001$)	$N = 9$

Low-grade: combination of hyperplasia and mild dysplasia.
High-grade: combination of moderate and severe dysplasia.

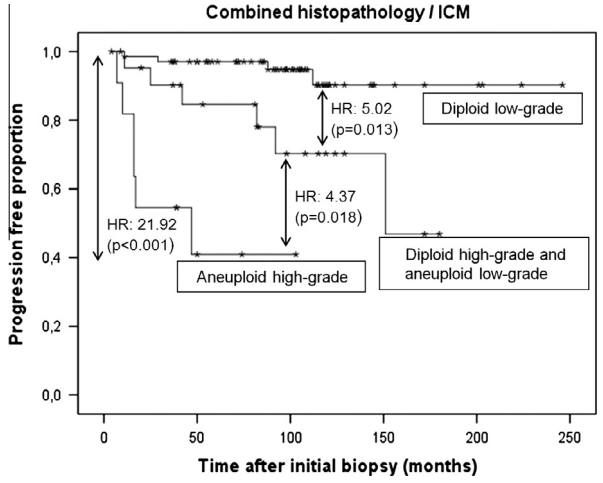

Figure 5. Outcome of the ICM combined with histopathological subclasses. Aneuploid low-grade combined with diploid high-grade: HR: 5.0 (CI: 1.41–17.89; $p = 0.013$) (adjusted to diploid low-grade). Aneuploid high-grade HR: 21.9 (CI: 5.78–83.18; $p < 0.001$) (adjusted to diploid low-grade). Aneuploid high-grade: HR: 4.4 (CI: 1.29–14.70; $p = 0.018$) (adjusted to aneuploid low-grade + diploid high-grade).

Table 6
Monitoring of related leukoplakia and malignant lesions that developed at the same location over time.

Patient	Initial premalignancy	Intermediate premalignancies	SCC	Intermediate premalignancies	Recurrent SCC(s)
1	A	D	D	A	A
2	A	AA	A		A
3	A	A	A[a] D	A[a]AA	A[b]
4	A	A	A		A[a]
5	D	D	D		
6	D		D	D[b]D[b]	
7	A	A	A	A	AA
8	A	A	A[a]		
9	D		D	D	

Sequential ploidy scores represent mucosal disorders developed subsequently.
A = aneuploid lesion; D = diploid lesion. All values represent the same scores for ICM and FISH except the cases marked with [a](ICM is aneuploid and FISH diploid).
[b] FISH data not available.

Table 7
Monitoring of three patients with multiple leukoplakia without malignant transformation.

Patient	Biopsy year	Biopsy site	Histopathologic diagnosis	ICM	FISH	Treatment
10.	**1999**	**FOM front**	**D+++**	**A**	**A**	**Laser**
	2000	FOM front	D+++	D	A	Laser
	2002	FOM front	D+++	D	D	Laser
	2006	End of follow-up				
11.	**2004**	**Buccal mucosa left**	**D+++**	**A**	**A**	**Surgery**
	2007	Buccal mucosa left	D+++	D	D	Expectative
	2010	End of follow up				
12.	**1997**	**Trig. Retromolare Right**	**D++**	**A**	**D**	**Expectative**
	1997	Trig. Retromolare right	HP	D	A	Expectative
	2001	Patient died because of heart failure				

FOM = floor of mouth; Trig = trigonum; HP = hyperplasia; D++ = moderate dysplasia; D+++ = severe dysplasia; D = diploid; A = aneuploid). Precursor lesions described in bold is the first biopsied lesion.

Discussion

In this study we analysed the added value of CI for the assessment of the malignant potential of oral leukoplakia, using two techniques (ICM and dual target FISH for chromosomes 1 and 7). Previous studies established that histopathology is only partly successful in predicting future behaviour of premalignant oral lesions [8,9,11,12]. Data of the present study show that CI assessed by the two different approaches is an independent prognosticator, providing valuable information in addition to the histopathological diagnosis. Combining these sources of information, three groups of leukoplakia possessing respectively low, intermediate and high risk for malignant transformation may be discriminated. Moreover, it was found that CI detected by ICM might be a useful tool to monitor lesions over time. Generally, successive aneuploid premalignancies ultimately resulted in (aneuploid) tumours, whereas a shift from an aneuploid to a diploid status suggested a favourable prognosis.

Our data concerning the prognostic value of the ploidy status of oral leukoplakia are largely concordant with existing literature

[14,16,17]. In the present study, aneuploid oral lesions showed malignant progression in 43.5–47.1% (detected by ICM and FISH, respectively). Similar percentages are found in other studies [14,16,17]. Also in line with these studies, not all aneuploid leukoplakia transformed into malignancies. The results might be explained by the fact that these studies (including the present study) are based upon retrospective data, harbouring different intervention modalities (both radical and irradical) which ranged from watchful waiting to surgical excision or laser ablation. As reported by Torres-Rendon et al. [14] we observed that aneuploid premalignancies are able to transform into SCC after a long time span (in this study one case even after 12.6 years). Also, the fact that diploid lesions may progress to SCC was repeatedly found [14,16,17].

In this study, 37.5% of the developed SCC were found to be diploid (FISH 56.3%), which may (at least partly) be explained by the assessment method used. Previous studies found a large variability in the percentage of diploid oral SCC (8–70%) [14,15,31,32]. The lowest number of diploid SCC (8%) was found using a very sensitive method (CV values as low as 1%) applying FCM [15]. In the present study using ICM, CV values of approximately 5% were found, making distinction between diploid and near-diploid populations hazardous. Okafuji et al. concluded that sensitive techniques such as comparative genomic hybridisation showed that all oral carcinomas were aneuploid, but that many are not detected as such by techniques as FCM or ICM because of their detection limit [33,35]. Another factor to consider when assessing ploidy status is heterogeneity within oral lesions as reported before [33,34].

Baretton et al. showed increased sensitivity for ICM based ploidy assessment, measuring 'in situ' in histological sections, rather than isolating whole nuclei from paraffin blocks [32]. Apparently, the possibility of selective sampling of nuclei in suspicious regions enables detection of small aneuploid subpopulations, even when applying a less sensitive measurement procedure. In the present study, three of the initial premalignancies were diploid in ICM but aneuploid in FISH. Besides sampling error an explanation might be the inability of ICM (on whole nuclei) to detect small aneuploid subpopulations in the biopsy specimen. In this study, FISH analysis, which is also applied to selective tissue section areas, identified in general less aneuploid cases compared to ICM, resulting in decreased sensitivity for predicting progression (with a corresponding increase in specificity). This may be explained by sampling bias or by the fact that FISH only assesses copy number variations of two centromere probes.

The results of Baretton et al. [32] are in accordance with our previously published data, in which ploidy assessment in tissue sections was combined with assessment of expression of biomarkers Ki67, p53 and yH2AX [10]. Despite a sufficiently high sensitivity of ICM and FISH also diploid precursor lesions as well as their subsequent tumours may be found. It has been shown previously that a minority of oral lesions follow an alternative genetic pathway, ultimately resulting in an invasive phenotype not harbouring large copy number alterations or chromosome instability [36]. This phenomenon may also explain the results found in the present study. Further study is therefore recommended. Combining ploidy data with analysis of biomarkers relevant for this alternative pathway even may yield additional information and increase sensitivity [10].

Subdivision of leukoplakia into three risk levels, based on the combined histopathology and CI analysis may lead to a more appropriate patient treatment. Both ICM as well as FISH are suitable for this approach. In this study, aneuploid high grade dysplasia (comprising 11% of cases) had a progression free survival of approximately 40% after 4 years (HR of 22.0 compared to low histological grade diploid cases). Similar results were published previously (HR of 19.3 for aneuploid high grade dysplasias, which constituted 8% of the population) [17]. In comparison, 37.5% of OSCC patients showed recurrence in a comparable time interval after treatment [37]. Therefore, considering these lesions to be similar to OSCC in terms of treatment options may be considered. On the other end, diploid low grade leukoplakia are largely devoid of malignant transformation (<3% after 6 years), justifying a 'wait and see' policy. Especially for cases of mild and moderate dysplasia, CI analysis may be used to tune clinical management. Results from this study suggest that an aneuploid mild dysplasia is comparable to a diploid moderate dysplasia in terms of progression free survival. On the other hand, a diploid mild dysplasia may be eligible for a 'wait and see' policy whereas an aneuploid moderate dysplasia may demand treatment comparable to an OSCC. In this way, CI analysis may greatly reduce the number of cases of intermediate malignant potential, which are inherently difficult to manage.

With regard to the option of using CI analysis for monitoring patients over time, promising results were obtained. Considering the low number of cases for which data were available, however, a prospective study is required to confirm this hypothesis.

In conclusion, assessment of CI in oral leukoplakia yields independent prognostic information, which may contribute to individual patient management. Different assays may be applied to establish the CI status, having their own merits and weaknesses. In the present study the combination of histopathology and CI (both ICM and FISH) improved the assessment of the malignant potential and may enable the monitoring of (treated) oral leukoplakia over time. CI analysis can help to select patients with a high risk of malignant progression and therefore may identify a subset of leukoplakia suited for more strict follow-up as well as a group of leukoplakia eligible for more aggressive treatment.

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