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Abnormal DNA content predicts the occurrence of carcinomas in non-dysplastic oral white patches

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Abstract

The majority of oral squamous cell carcinomas (OSCCs) are preceeded by visible changes in the oral mucosa, most often white patches. Although the histological finding of dysplasia in oral white patches signals increased risk of developing OSCC, this may also occur in non-dysplastic lesions. However, no reliable markers exist to predict the occurrence of OSCC in these patients. From a total of 263 patients diagnosed with oral white patches, biopsies from 45 patients were selected on the criteria that the patients had lesions histologically proven to be non-dysplastic. The lesions were analyzed with respect to their DNA content. The clinical outcome of the patients was known from the Cancer Registry of Norway, and these data were compared to the DNA content of their lesions. Among the 45 patients, five cases (11%) later developed an OSCC. Four of the cases that subsequently developed an OSCC were among the five aneuploid (abnormal) cases ($P=0.001$). One aneuploid lesion did not develop a carcinoma during a follow-up time of 120 months. The fifth case that subsequently developed an OSCC was diploid (normal), and developed into an OSCC after an observation time of 73 months ($P=0.001$). In conclusion, aberrant DNA content reliably predicts the occurrence of OSCC in patients that otherwise would be regarded as at very low risk. Normal DNA content indicates low risk.

Keywords: DNA ploidy; Image cytometry; Histological diagnosis; Prognostication; Oral white patches

1. Introduction

White patches are the most commonly encountered visible oral mucosal lesions [1] and are associated with subsequent occurrence of oral squamous cell carcinomas (OSCC) [2,3], which show an increasing incidence, particularly in young persons [4]. However, no currently established method accurately predicts the clinical outcome of oral white lesions [5,6].

Strong evidence points to aberrant DNA content in cells (DNA aneuploidy) as a cause rather than as a consequence of malignant transformation [7]. Several studies indicate that mutations in genes controlling chromosome segregation during mitosis and centrosome abnormalities play a critical role in causing chromosome instability in cancer [8–10]. Furthermore, chromosomal aberrations consistent with impaired fidelity of chromosome segregation during mitosis have been shown to occur exclusively in aneuploid tumor cell lines [11], pointing to a central role of aneuploidy in carcinogenesis [12]. DNA content (DNA ploidy) has also been found to be a factor with prognostic impact in precancers or early stage carcinomas of the head and neck [13,14]. Finally, a gradual increase in quantitative DNA aberrations has been found to correlate with increasing degree of dysplasia in oral mucous membranes [15]. If patients at risk could be identified by analysis of parameters that reflect the true biological properties of the lesions, treatment regimes could be given selectively [16] to improve the currently poor 5-year survival rate of OSCC [17–19].

Although reliable predictors of the clinical outcome of patients with oral white lesions are not established, the histological finding of dysplasia is associated with increased risk of developing OSCC [20]. In accordance with this, we have previously investigated the prognostic

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impact of DNA content in dysplastic oral white patches, and found that the long-term outcome of patients with such lesions is predicted with a considerable degree of certainty by measuring the DNA content of such lesions [21]. However, there is a morphological continuum from normal epithelium to carcinoma, and the distinction between dysplastic and non-dysplastic lesions is not clear [22,23]. Thus, DNA content may reflect genomic instability of cells very early in the malignant process, at a point where morphological changes in malignancies or premalignancies are not yet evident. Conceivably, lesions that are defined as non-dysplastic, and therefore, considered to represent minimal risk for developing OSCC, may contain readily detectable large scale genomic alterations (DNA aneuploidy) which could signal a malignant transformation [22].

To investigate whether alterations in DNA content predict the occurrence of OSCC in non-dysplastic oral white patches, we have estimated their DNA content using a high resolution image cytometry system and compared this to the clinical course in 45 patients, which according to four experienced pathologists all had non-dysplastic lesions.

2. Patients and methods

Biopsies from a total of 263 patients clinically diagnosed with oral white lesions between 1976 and 1995 were obtained from the archives of the Department of Pathology, University of Bergen and Department of Pathology and Forensic Odontology, University of Oslo. Biopsies from 45 of these patients, 28 males (mean age [±S.D.] 64.8±12.9 years, range 43.9–79.2) and 17 females (mean age [±S.D.] 58±11.4 years, range 44.5–78.1) were histologically proven as non-dysplastic by four experienced pathologists. The DNA content (DNA ploidy) of these lesions were measured. SNOMED codes for the localization of the biopsies and the following carcinomas were used to verify close spatial relations between the white lesions and subsequent carcinomas. The DNA ploidy analysis was performed on tissue from the same blocks as the histological assessment had been performed.

Patients with previous or concomitant clinical features of erythroplakia were not included in our study population, as erythroplakias generally are associated with a high occurrence of cancer [24,25]. Patients previously or simultaneously diagnosed with carcinoma in situ or carcinomas of the oral cavity were also excluded from the survival analysis, as these patients are prone to developing a secondary carcinoma [26]. Given that multiple lesions may arise due to widespread migration of transformed cells through the aerodigestive tract [27,28], patients with previous or concomitant tumors of the upper aerodigestive tract were also excluded.

Prognostic data were available from the Cancer Registry of Norway and could be compared to DNA profile of the lesions. The minimum follow-up time for this group of 45 patients was 63 months.

2.1. DNA cytometry

Biopsies fixed in 4% buffered formaldehyde and paraffin embedded were sectioned. Two 50 μm sections were cut and enzymatically digested (SIGMA protease, type XXIV, Sigma Chemical Co., St. Louis, Missouri, USA) for the preparation of isolated nuclei (monolayers) according to the method of Hedley [29]. The nuclei were stained by the Feulgen-Schiff method [30] and DNA ploidy analysis was performed by image cytometry according to an established protocol [31].

The Fairfield DNA Ploidy System (Fairfield Imaging Ltd., Tunbridge Wells, UK) was used for measurements and analysis. This image processing unit consisted of a Zeiss photomicroscope equipped with a 40/0.75 objective lens (Zeiss), a 546-nm green filter and a black and white CCD camera (C4742-95, Hamamatsu Photonics K.K., Hamamatsu, Japan). The analyzed images consisted of 1024×1024 pixels, with a 10-bit resolution per pixel, i.e. 1024 possible grey level values for every pixel. The final resolution was 1600×, with an estimated pixel size of 0.2 microns. At least 300 cell nuclei were measured and stored in galleries for each case, and lymphocytes (up to 30) were included as internal controls. Sampling of the epithelial cell nuclei was done by a specially trained observer (see Acknowledgements).

The rules for classification were as follows: a lesion was classified as diploid if only one G0/G1 (2c) peak was present or if the number of nuclei in G2 (4c) peak did not exceed 10% of the total number of epithelial nuclei, or if the number of nuclei with a DNA content >5c did not exceed 1% of the total number of epithelial cells (Fig. 2). A lesion was defined as tetraploid when its G0/G1 (4c) peak was present together with its G2 peak (8c) or when the fraction of nuclei in the tetraploid region exceeded 10% of the total number of nuclei, without corresponding S-phase. A lesion was defined as aneuploid if non-euploid peaks were present or the number of nuclei with a DNA content >5c or 9c exceeded 1%. The mean coefficient of variation (CV) of the diploid peak for all the 45 cases (diploid, tetraploid and aneupolid) was 5.7%, range 3.3–7.9%. The corresponding number for the diploid cases are 4.6%, range 3.6–6.7%.

2.2. Statistics

The actuarial probability of disease-free survival was calculated according to the method of Kaplan and Meier. Comparison of groups was done by the log-rank test. The following factors were entered into Cox

multivariate analysis: age, sex, size and multifocality of the lesions (one lesion vs. two or more) as well as exposure to risk factors (in particular, tobacco and alcohol, not further quantified). Comparison of the groups (diploid, tetraploid and aneuploid) was done by the Fisher's exact test for categorical data. All statistical tests were two-sided, and values less than 0.05 were considered to indicate statistical significance. All calculations were performed with SPSS statistical software (version 9.1, SPSS, Chicago).

3. Results

3.1. Characteristics of the patients

Clinical data were available for all the 45 patients included in the study, and the characteristics of the study population is shown in Table 1.

3.2. Diagnostic range in the typing of the lesions (n = 45)

A total of six different diagnoses (lichen ruber planus, hyperplasia, achantosis, normal mucosa, chronic inflammation and integument) were made, with quantitative distribution as shown in Table 2.

The most common anatomic sites for the lesions were the buccal mucosa (12), hard palate (eight), and the sublingual region (25). With a possible exception of lichen ruber planus, none of these entities are considered to have a significantly increased malignant potential [32].

Table 1
Characteristics of patients (as reconstructed from the medical records)[a]

Variable	Number of patients
Female/male ratio	26/19
Previously diagnosed leukoplakia	35
Size of white patches (largest diameter, in cm)	
<1	3
1–2	18
2–3	6
>3	18
Multifocality	
No	18
Yes Unilateral	17
Bilateral	10
Smoking status	
No tobacco habits	11
Previous tobacco habits	18
Current tobacco habits	16
Use of alcohol	
Yes	35
No	10

[a] Mean age (year), 70.3.

3.3. DNA ploidy classification in relation to clinical outcome

Thirty-five lesions (78%) were classified as DNA diploid, five lesions (11%) were classified as tetraploid and five lesions (11 %) were classified as aneuploid (Table 3). Of the 35 diploid cases, one later developed an OSCC (3%) and the carcinoma was diagnosed 37 months after the initial biopsy. This patient was still alive after an observation time of 93 months. The biopsy was from a unifocal lesion. From the DNA ploidy classification, however, one would expect all the 35 diploid cases to have a favorable clinical outcome. Indeed, this is the case for 34 out of 35 diploid (97%) cases with normal DNA content.

Altogether, five cases were classified as aneuploid. A priori, these cases would have an unfavorable prognosis. Indeed, four cases later developed a carcinoma (80%) (Table 3). One case had a unifocal lesion, two cases had two lesions, of which one had bilateral lesions, and one had three lesions bilaterally distributed in the floor of the mouth. The fifth case had a follow-up time 120 months without developing a carcinoma. This case had biopsies taken from two lesions. Of the four patients that developed a carcinoma, three patients later died of their disease. All the carcinomas occurred in the same location as the preceeding oral white patch, as confirmed by SNOMED codings. The Kaplan-Meier curves for the 45 cases of non-dysplastic lesions is shown in Fig. 1.

3.4. The diagnostic range of the five cases that developed OSCC

The diagnostic range of the four cases that developed a carcinoma is shown in Table 4. The four observers diagnosed the diploid lesion either as normal mucosa

Table 2
The range of diagnoses in 45 oral leukoplakias assessed by four independent observers (A, B, C and D)

	A	B	C	D	Number of cases common to at least two observers
LRP[a]	2	3	1	2	2
Normal	12	13	18	14	9
Chr. infl.[b]	17	13	11	12	7
Hyperplasia	12	12	15	14	9
Integument	0	1	0	1	0
Achantosis	3	4	1	3	1
Total	45	45	45	45	28

[a] Lichen ruber planus.
[b] Chronic inflammation.

Table 3
The relationship between DNA ploidy classification and histological typing made by four separate pathologists (A, B, C and D) in 45 cases of non-dysplastic leukoplakias[a]

DNA ploidy	No of cases	Histological typing											
		Normal		Hyperplasia		Chr. infl.[b]		Achantosis		LRP[c]		Integument	
Diploid	35	A[d] **10**	B **10**	**9 (1)**[e]	**10**	**14**	**10 (1)**	**2**	**2**	**1**	**3**	**0**	**1**
		C **16 (1)**	D **11**	**10**	**11 (1)**	**9**	**9**	**0**	**2**	**1**	**2**	**0**	**1**
Tetraploid	5	**1**	**1**	**2**	**1**	**1**	**3**	**0**	**0**	**1**	**0**	**0**	**0**
		1	**2**	**3**	**2**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
Aneuploid	5	**1 (1)**	**2 (1)**	**1 (1)**	**1 (1)**	**1 (1)**	**0**	**1**	**1 (1)**	**0**	**0**	**0**	**0**
		2 (1)	**1 (1)**	**0**	**1**	**1 (1)**	**1 (1)**	**1 (1)**	**1 (1)**	**0**	**0**	**0**	**0**

[a] DNA ploidy as measured in the 45 cases that were classified as non-dysplastic. For each observer (A, B, C and D), the cases have been allocated to a histological diagnosis and a DNA ploidy classification. Numbers in bold indicate the number of cases with a particular histological diagnosis and DNA ploidy classification for each observer (A, B, C and D). Of the 45 non-dysplastic cases, 35 were classified as diploid, of which one have later developed a carcinoma. There were five aneuploid cases, of which four later developed a carcinoma. None of these cases had been given a histological diagnosis with an established or putative malignant potential. Five cases were classified as tetraploid, of which none later have developed a carcinoma.

[b] Chronic inflammation.

[c] Lichen ruber planus.

[d] The letters A, B, C and D denote the four observers. The same pattern is repeated for all quadruples of squares allocated to DNA ploidy groups and histopathological diagnosis.

[e] Numbers in brackets denote the cases that later developed a carcinoma.

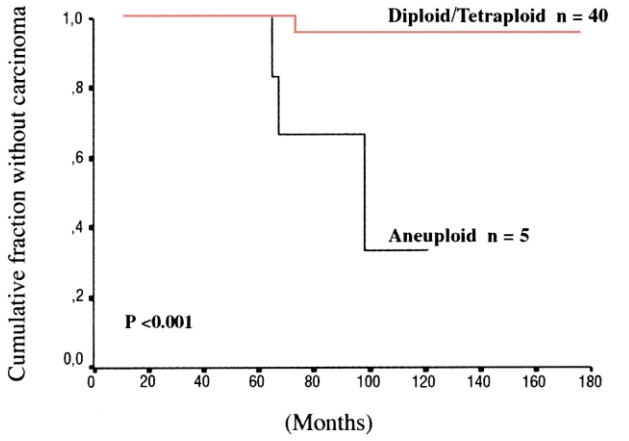

Fig. 1. Disease free survival as related to nuclear DNA content (DNA ploidy) in 45 cases of oral non-dysplastic oral white patches. Only the clear cut groups of diploid and aneuploid are considered. In the group of five tetraploid lesions, none acquired a carcinoma. Of a total of 35 diploid cases, only one case (3%) developed a carcinoma (74 months after the initial diagnosis) and of a total of five aneuploid cases, four (80%) later developed an OSCC (61, 63, 98 and 98 months after the initial diagnosis).

(Observer C), chronic inflammation (Observer B) or hyperplasia (Observers A and D). For the aneuploid lesions the diagnostic range is similar, with an addition of achantosis for observers B, C and D. The DNA ploidy histograms for three of the four aneuploid cases and the one diploid case that later developed a carcinoma are shown in Fig. 2.

3.5. Statistical tests

Table 5 shows a 2×2 table displaying the clinical outcome as related to DNA ploidy group. The two-tailed *P*-value indicates a statistically significant difference between the good prognosis groups (diploid) and poor prognosis group (aneuploid) with regard to clinical outcome ($P = 0.001$). The actuarial disease-free survival for the prognostic groups (diploid/normal and aneuploid/abnormal) are shown in the Kaplan–Meier curves in Fig. 1 ($P < 0.001$). There was no prognostic value of age, sex, multifocality of the lesions and exposure to risk factors (data not shown).

4. Discussion

OSCCs currently have a poor 5-year survival rate [17–19], most likely attributable to the fact that the carcinomas are disseminated at the time of a reliable diagnosis [18,33], making curative measures less effective.

In Norway, personal identification numbers and updated national registers for premalignant and malignant lesions as well as place of residency offers the opportunity to monitor patients with complete data on morbidity and mortality. In this retrospective study we found that in a total of 45 oral white lesions histologically proven to be non-dysplastic and without a malignant potential based on histological typing, five cases later developed OSCC at the same anatomical site as the preceding white patches. The histological typing of

Table 4
Histological diagnosis from assessing HE stained sections in the five cases that later developed an OSCC (one diploid and four aneuploid cases)[a]

	Observers			
	A	B	C	D
Diploid case (one)	Hyperplasia	Chr. infl.[b]	Normal	Hyperplasia
Aneuploid cases (four)	Normal	Normal	Normal	Normal
	Hyperplasia	Hyperplasia	Chr. infl.	Chr. infl.
	Chr. infl.	Achantosis	Achantosis	Achantosis
	Achantosis	Hyperplasia	Normal	Chr. infl.

[a] The diagnostic range of the five cases that developed a carcinoma is shown in Table 3. The four observers type the diploid lesions either represent normal mucosa (Observer C), chronic inflammation (Observer B) or hyperplasia (Observers A and D). For the aneuploid lesions the diagnostic range is similar, with an addition of achantosis for observers B, C and D.
[b] Chronic inflammation.

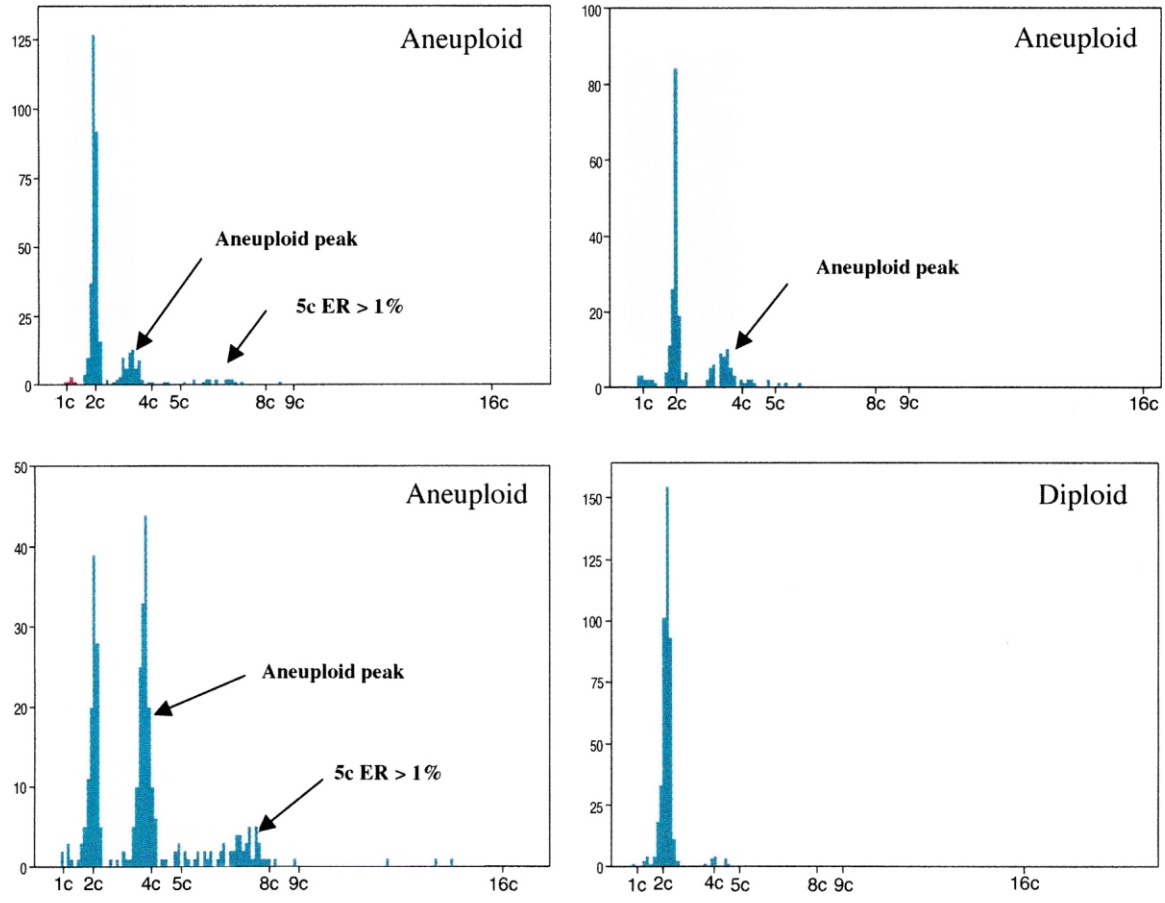

Fig. 2. DNA ploidy histograms of four of the five presumed non-malignant lesions that later developed a carcinoma in the same location as the original biopsy was obtained. The arrows indicate either an aneuploid peak. 2c indicated the diploid peak, 4c the tetraploid peak and 8c the octaploid peak. The red columns given in the upper left panel are reference cells.

the non-dysplastic lesions was performed independently by four experienced pathologists. Other than the white patches, there were no other clinical features of the patients that would indicate an increased risk for developing OSCC, such as the presence of erythroplakias, previous carcinomas of the oral cavity or previous or concomitant cancers of the upper aerodigestive tract. Four of these five cases were identified by measuring DNA content by high resolution image cytometry. One of the five cases that subsequently developed OSCC would not have been detected with our methods, as it was classified as diploid (normal) in our study. However, the finding of normal DNA content (diploidy) indicates a low risk for subsequent development of a

Table 5
Incidence of carcinomas as related to DNA ploidy status at the time of initial diagnosis[a]

Clinical status			
DNA ploidy	No subsequent development of carcinoma	Subsequent development of carcinoma	P-value[b]
Di-/Tetraploid	39 (98%)	1 (2%)	0.001
Aneuploid	1 (20%)	4 (80%)	

[a] A 2×2 table showing the clinical outcome of 45 non-dysplastic cases as related to DNA ploidy groups (diploid and aneuploid). Of the 35 cases classified as diploid, only one case later developed a carcinoma (after an observation time of 37 months). Of the five cases classified as aneuploid, four cases later developed a carcinoma. The fifth aneuploid case has not developed a carcinoma during a follow-up time of 120 months.
[b] Two-tailed.

carcinoma. Interestingly, none of the five cases that later developed a carcinoma were diagnosed as lichen ruber planus, which is the only one of the lesions listed in Table 2 with a putative malignant potential, although data on this particular entity are controversial [34]. The histological diagnoses given by the four observers were significant contributions to the planning of treatment and follow-up in these patients. Therefore, these observations may be said to be false negative with respect to predicting the clinical outcome of these patients.

DNA ploidy measurements by image cytometry is a relatively crude method, with a coefficient of variation of 3–5%, which translates to 1–2 chromosomes per nucleus. However, the long follow-up time in our data supports the notion that DNA content is a highly sensitive and specific marker for predicting the subsequent occurrence of an OSCC in the study patients. Emerging evidence may serve to explain these findings. The analysis of a large number of solid tumors of epithelial origin has invariably revealed a tumor specific pattern of chromosomal gains and losses. Ried et al. [9] showed that aneuploid lesions on average have a higher number of chromosomal imbalances (termed average number of copy alterations, ANCA) per cell line than diploid lesions. Ghadimi et al. [11] recently demonstrated that centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines. The relation between genetic alterations and aneuploidy may be explained by mechanisms involving the spindle apparatus and centrosomes [9,35] and in oral cancer cells, gross numeric chromosomal aberrations have been shown to be the result of cytoskeletal defects and breakage-fusion-bridge cycles in the mitotic apparatus or kinetochore [8]. Finally, a gradual increase in large-scale DNA aberrations has been found to correlate with an increasing degree of dysplasia in oral mucous membranes [15] and studies have shown that genetic instability, as measured by the number of chromosomal copy alterations per case, increase significantly at the transition from precursor lesions to invasive cancer and with tumor agressiveness [36].

The subsequent occurrence of a carcinoma in a diploid case may have several explanations. The obvious explanation would be a sampling error. Conceivably, an aneuploid cell clone could be localized in the vicinity of the biopsy without being included in it. However, this patient had a unifocal white patch, from which the biopsy was obtained. It seems unlikely that an aneuploid cell clone should be localized in the neighboring, apparently normal mucosa, but not within the white patch. Perhaps a more likely explanation would be that genetic changes leading to malignant transformation may lead to only small quantitative genomic alteration, too small to be detected by our methods. One patient with an aneuploid lesion did not develop an OSCC during an observation time of 120 months. Although this is a substantial follow-up time, a subsequent malignancy cannot be entirely ruled out. The identification of epithelial cell nuclei from a monolayer preparation may pose a problem with respect to morphological control, as the native tissue structure is disrupted and the cell nuclei are not surrounded by cytoplasm. However, in oral epithelium, the size of epithelial cell nuclei by far exceeds that of stromal and inflammatory cells, as the diameters of the epithelial cell nuclei are in the range of 10–15 μm (unpublished data). Should stromal cells have been included, they most likely would be diploid or even tetraploid, but not aneuploid. Stromal cells would therefore represent a diluting effect on a possible aneuploid population of cells and accordingly would reduce the sensitivity of the DNA ploidy analysis with respect to detecting aneuploid lesions. However, the high positive predictive value of aneuploidy and the corresponding negative predictive value of diploidy with respect to subsequent occurrence of OSCC indicates that the possible adverse effect of including stromal or inflammatory cells is negligible.

The size of the patches most likely influence the prognosis of the white lesions [37]. However, we have not been able to reliably reconstruct the size of the lesions, neither from the biopsy blocks nor from the medical records. Accordingly, the prognostic impact of this parameter has not been investigated in the present study.

Considering the limited number of cases presented, inferences from the present study should be drawn with caution, and ultimately the clinical value of measuring DNA content in oral premalignancies must be evaluated

from a prospective trial. Nevertheless, the presented data represent evidence that measurements of DNA content give valuable prognostic information that may serve to identify and treat patients with oral mucosal lesions that otherwise would be regarded as without a malignant potential. It may not be feasible to screen all patients with oral white patches on a regular basis. However, high-risk patients (tobacco-users, alcohol abusers, patients with erythroplakias) who are willing to come to one examination could be followed, e.g. by measuring DNA content. Intensive counseling, non-invasive interventions such as chemoprevention or photodynamic therapy and follow-up efforts could then be offered to patients who harbor lesions with aberrant DNA content.

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