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DNA ploidy measurement in oral leukoplakia: Different results between flow and image cytometry

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SUMMARY

The estimated prevalence of oral leukoplakia is worldwide approximately 2%, with an annual malignant transformation rate of approximately 1%. The aim of the present study was to evaluate the possible contribution of ploidy measurement to the prediction of the clinical course, in a well defined cohort of patients with oral leukoplakia. Ploidy was measured by both flow cytometry (FCM-DNA) and image cytometry (ICM-DNA) and we focussed on the comparison of the two different techniques to determine ploidy. A total of 41 patients have been included, with a mean age of 59 years (range 36–78 years). With FCM-DNA, three lesions were aneuploid, with ICM-DNA, 19 lesions were aneuploid. DNA ploidy was compared with clinicopathological and patients parameters. There were no statistically significant differences between DNA ploidy and any patient factor with both FCM-DNA and ICM-DNA. Using FCM-DNA, DNA aneuploid lesions showed statistically significant more dysplasia ($p = 0.04$) than diploid lesions. Furthermore, DNA aneuploid lesions were more frequently encountered at high-risk locations ($p = 0.03$) as being determined with FCM-DNA. These relations were not found when DNA ploidy was determined with ICM-DNA.

Introduction

Leukoplakia is defined as 'a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer'.[1] The estimated prevalence of oral leukoplakia worldwide is approximately 2%,[2] with an annual malignant transformation rate into oral squamous cell carcinoma (OSCC) of approximately 1%.[3] Several factors are known to be associated with an increased risk of malignant transformation of leukoplakia, e.g. homogeneity and size of the lesion.[3–5] At present, the best predictor is the presence of epithelial dysplasia. Nevertheless, some dysplastic lesions may remain unchanged or resolve over time.[6,7] A limitation of dysplasia grading is its relative subjective character, indicated by a high inter- and intra-examiner variability in the assessment of dysplasia.[8–11]

Another suggested parameter of prognostic value could be DNA ploidy measurement.[12] DNA aneuploid lesions in Barrett's esophagus have shown a higher risk of malignant transformation[13] and DNA aneuploid gastric cancer has an unfavourable prognosis compared to DNA diploid cancer.[14] Recent DNA ploidy studies of dysplastic oral lesions have suggested that DNA aneuploid lesions carry a higher risk for OSCC progression.[15–18] The DNA ploidy status can be measured by either flow cytometry (FCM-DNA) or image cytometry (ICM-DNA).

The aim of the present study was to evaluate the possible difference between DNA ploidy measurement using either FCM-DNA or ICM-DNA, in a well defined cohort of patients with oral leukoplakia. This study is a continuation of the study of Bremmer et al.[18] Relations between DNA ploidy and the histopathological grading and other clinical parameters, such as gender, smoking habit, alcohol consumption, homogeneity, size and location of the lesion were investigated. Moreover, all leukoplakias were classified by the OL-classification and staging system.[3]

Material and methods

Patients

For the purpose of this retrospective study, 87 patients were selected on the basis of biopsied leukoplakias with sufficient material for histopathological grading as well for DNA ploidy measurement,

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and a minimum follow-up time of 12 months. All patients were referred to the Department of Oral and Maxillofacial Surgery at VUmc/ACTA, Amsterdam, and the biopsies were taken between 2003 and 2010. Patients with an (previous or concomitant) OSCC were excluded ($n = 8$). Also, patients in whom DNA ploidy could not be determined were excluded ($n = 38$). A total of 41 out of 87 patients fulfilled all inclusion criteria: 20 men and 21 women, with a mean age of 59 years (range 36–78 years). Nine patients in this cohort overlapped between this study and the study by Bremmer et al.[18] The smoking habits and alcohol consumption of these patients are shown in Table 1.

Clinically, a distinction was made between homogenous and non-homogeneous leukoplakia. There were 28 patients with a homogeneous leukoplakia and 13 with the non-homogeneous type. The location of leukoplakia was specified according to six sites: tongue, floor of mouth (FOM), hard palate, buccal mucosa, upper and lower alveolus and gingiva, and multiple sites (Table 2). All this information was extracted from the patient files.

The size, presence and grade of epithelial dysplasia were determined by means of the OL-classification and staging system (Table 3).[3] Twenty-three leukoplakias were L_1, 10 cases were L_2 and seven were L_3. Size could not be determined in one case. There were 23 excisional and 18 incisional biopsies performed. All biopsies were revised on haematoxylin and eosin (H&E) stained sections by the same investigators (EREAB/EB), a joint consensus was made for the cases that were graded differently. Severe dysplasia (P_2) was diagnosed in six biopsies, mild-moderate dysplasia (P_1) in 14 biopsies, while dysplasia was absent (P_0) in 21 biopsies. The biopsy on which the histological grading was done, was also used for DNA ploidy determination.

Initial and follow-up management included surgical excision, CO_2-laser therapy or observation. All patients were advised to quit smoking.[19] The follow-up period ranged from 12 to 87 months with a median of 20 months. The follow-up period started at the date the biopsy was taken. It ended in case of lost to follow-up, death, or development of OSCC at the site of oral leukoplakia or elsewhere in the oral cavity.

The design of this study adheres to the code for proper secondary use of human tissue of the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org).[20]

Tissue processing for FCM-DNA and ICM-DNA

Formalin fixed paraffin-embedded tissue specimens, the same which were used for histopathological grading, were collected from the pathology archive. Two or three 50 μm sections were cut from the tissue specimens and nuclear cell suspensions were prepared according to the Hedley procedure.[21] For FCM-DNA, part of the nuclear suspension was stained with DAPI (4′,6-diamidino-2-phenylindol, Partec Instruments, Muenster, Germany). For ICM-DNA, cytospins were prepared from the other part by centrifugation of the specimen for 15 min at 3000 rpm. The cytospins for ICM-DNA were stained using the Feulgen method according to the consensus of the European Society for Analytical Cellular Pathology,[22] with minor modifications. Cytospin preparations were placed in 5 N HCL for 30 min at 27 °C. Hereafter the cytospins were rinsed in distilled water for 5 min, stained with fresh Schiff's reagent for 45 min, and then washed in running tap water for 15 min. Last, the cytospin slides were dehydrated and cover slipped.

Table 2
Site distribution of 41 patients with leukoplakia.

Oral subsite	Number of patients
Tongue (dorsal and lateral surfaces)	10
Floor of mouth	7
Hard palate	3
Cheek mucosa	5
Upper and lower alveolus and gingiva	4
Multiple sites	12
Total	41

Measurement of FCM-DNA

The FCM-DNA measurements were done within 3 h after DAPI staining. For the measurements a Partec Pas II mercury lamp-based flow cytometer (Partec Instruments) was used. Trout erythrocytes were used as external control cells. The procedure is described in detail elsewhere.[23]

Measurement of ICM-DNA

The DNA content of stained nuclei was measured and analysed by ICM-DNA according to a published protocol.[14,24] The guidelines of the consensus report of the European Society for Analytical Cellular Pathology were followed.[22] Köhler illumination was applied, before every ICM-DNA analysis. The camera was switched on at least 15 min before every measurement to ensure standardised conditions. Images were linearly corrected for shading with two empty images, namely one illuminated and one dark-current image.[25] The resulting corrected grey values provided a measure for the local optical density. Segmentation was carried out in a fully automatic manner.[14,24] A filter was used during measurements to remove debris and aggregates.

Approximately, 1000 nuclei were measured in a fully automatic manner. Lymphocytes and fibroblasts were included as internal DNA diploid controls and were used to calibrate and scale the DNA histogram. Using classification algorithms, round dark condense objects resembling lymphocytes and ellipsoid objects resembling fibroblasts were automatically identified. The majority of remaining debris and aggregates were automatically removed with another set of classification algorithms from the data set. The resulting DNA histograms were visually inspected. Nuclei, which should have been removed on the basis of features such as shape and texture automatically, but were missed by the classification algorithms, were removed manually.

In DNA cytometry, nuclear DNA content is measured in relative units 'c', in which the DNA content of normal nuclei is set at 2c. In this study, the 2c reference value was determined by taking the mean DNA content measured for nuclei that were identified as lymphocytes and fibroblasts. After establishing the 2c reference value, the histogram was scaled up to 10c with a fixed number of 256 bins to obtain standardised histograms that cover a wide

Table 1
Distribution of tobacco and alcohol habits among 41 patients with oral leukoplakia.

Gender	Patients	Tobacco			Alcohol		
		Smoker	Non-smoker	Unknown	Use	No-use	Unknown
Men	20	12	6	2	10	4	6
Women	21	14	5	2	6	4	11
Total	41	26	11	4	16	8	17