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Prognostic value of DNA ploidy status in patients with oral leukoplakia

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SUMMARY

Oral leukoplakia is a potentially malignant disorder that will develop into oral cancer at an estimated rate of 1–2% per year. Aim of the present study is to assess the possible predictive value of DNA ploidy for malignant progression of oral leukoplakia.

A cohort of 62 leukoplakia patients was studied and their biopsy was examined with standard histopathology and DNA image cytometry. Cox regression analysis was performed to establish the relationship between progression-free survival and the DNA ploidy status.

During the follow-up time (median of 69 months) 13 patients developed an oral squamous cell carcinoma (OSCC). DNA aneuploidy was observed in 27 (44%) patients and was significantly associated with a shorter progression-free survival [Hazard ratio of 3.7, 95% confidence intervals (CI) of 1.1 and 13.0 and a *p*-value of 0.04]. Sensitivity and specificity scores were 54% and 60%, respectively. Aneuploidy was not correlated with dysplasia grading (chi-square analysis).

DNA aneuploidy in oral leukoplakia is associated with an increased risk of progression to OSCC. However, for the individual leukoplakia patient, DNA ploidy status as single biomarker has limited value to predict progression to cancer.

Introduction

Oral cancer is a significant public health threat accounting for 270,000 new cases annually worldwide.[1] The majority of all oral cancers are squamous cell carcinomas (OSCC) that arise in the mucosal linings. Early diagnosis and treatment of OSCC may have a major impact on survival and quality of life. Clinically, oral potentially malignant disorders may appear as a white or a red lesion, designated as leukoplakia or erythroplakia, respectively. It has been estimated that approximately 1–2% of all leukoplakias progress into cancer per year.[2] Histopathological assessment and grading of epithelial dysplasia, if present, carry some subjectivity and, therefore, have limited value to predict the malignant potential in the individual patient.[3–5]

Earlier studies, mainly based on the measurement of allelic imbalance (AI), have shown that genetic analysis is promising in predicting malignant progression of leukoplakia. The presence of cells with AI at 3p, 9p and 17p has been shown to be associated with an increased risk for malignant transformation.[6–8] Limitations of measuring AI are the relatively high workload and the low analytical sensitivity at the assay level; for reliable measurement of AI at least 50% of the cells must be genetically altered,[9] necessitating enrichment of the preneoplastic areas by microd issection.

Abnormal nuclear DNA content, DNA aneuploidy, is an indicator of numerical chromosomal changes and its emergence is often a critical step in carcinogenesis.[10] DNA aneuploidy can be measured in a relatively robust and sensitive assay,[11] though lately its reputation as a marker of progression has been questioned.[12] DNA ploidy status can be measured either by flow cytometry (FCM-DNA) or image cytometry (ICM-DNA). ICM-DNA has several advantages over FCM-DNA as it allows for visual control and selection of specific cells for analysis based on morphological or additional features such as cell shape and texture. In addition, ICM-DNA permits additional measurements, because the specimen is a fixed cell suspension deposited on a glass slide, that can be stored after measurement.[13]

Two recent retrospective case–control studies have shown that DNA aneuploid oral dysplastic lesions have a higher risk of malignant progression and that ICM-DNA might help to identify those lesions at increased risk.[14,15] The aim of the present study is to determine the prognostic value of the DNA ploidy status in a cohort of patients with oral leukoplakia, in comparison with histopathological grading. ICM-DNA was used to analyze the DNA index and the clinical endpoint was development of OSCC.

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Patients, materials and methods

Patients

In this study 62 leukoplakia patients, who visited the outpatient clinic at the Oral and Maxillofacial Surgery Department of the VU University Medical Center in the period 1990–2010, were enrolled. Inclusion criteria were presence of leukoplakia defined according to Schepman et al.[16] and availability of sufficient representative formalin fixed paraffin embedded biopsy material for histopathological grading as well as for DNA ploidy measurement. This cohort can be considered as an unselected and unbiased group of leukoplakia patients. The median follow-up time of all patients was 69 months (range 10–193). Dysplasia was scored in hematoxylin and eosin-stained slides by an experienced pathologist according to the standard criteria of the World Health Organization[17] and was classified as no, mild, moderate or severe. Information on tobacco consumption was collected, and patients were divided in two groups: never *versus* former plus current smokers. A smoker was defined as having smoked more than five packyears in his or her lifetime. A packyear is a cumulative measure of tobacco smoking and reflects the consumption of a packet of cigarettes per day in a year. The occurrence of OSCC was confirmed by histopathological examination. Characteristics of the study population are summarized in Table 1.

The study was approved by the Institutional Review Board of the VU University Medical Center, and carried out according to the Dutch guidelines for experimental use of tissue samples.

Table 1
Patient and leukoplakia characteristics.

Variable	Number
Gender	
Female	40
Male	22
Age (years)	
Average	56
Minimum	24
Maximum	88
Tobacco consumption	
Current and former	43
Never	19
Treatment	
No	11
Yes	51
Surgery	34
Laser/surgery	6
Laser	11
Type of biopsy	
Incisional	30
Excisional	32
Site lesion	
Tongue	26
Non-tongue	36
Cheek	13
Floor of mouth	13
Alveolar ridge	8
Hard palate	1
Soft palate	1
Dysplasia	
No	35
Mild	16
Moderate	7
Severe	4

Tissue processing for ICM-DNA

From the formalin fixed paraffin-embedded tissue specimens, two or three 50-μm sections were cut and cell suspensions were prepared according to the Hedley procedure.[18] Cytospins were prepared, by centrifugation for 15 min at 3000 rpm, and stained by the Feulgen procedure according to the consensus protocol of the European Society for Analytical Cellular Pathology.[19] In short, cytospins were placed in 5 N HCl for 30 min at 27 °C, rinsed in distilled water for 5 min, stained with fresh Schiff's reagent for 45 min, and washed in running tap water for 15 min.

Measurement of ICM-DNA

DNA content of the stained nuclei was measured and analyzed by ICM according to an established protocol.[13,19,20] Approximately 1000 nuclei were measured in a fully automatic manner. Using classification algorithms, round dark condense objects resembling lymphocytes were automatically identified, as well as objects having an elongated oval shape representing fibroblasts. These objects were used as internal controls and to calibrate and scale the DNA-histogram.[13] Another set of classification algorithms was applied to automatically remove the majority of remaining debris and aggregates from the data set. The resulting DNA histograms were visually inspected and nuclei that 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.

Analysis of DNA-histogram

By convention in DNA cytometry, nuclear DNA content is measured in relative units '*c*', in which the DNA content of normal, non-cancerous nuclei is set at 2*c*. In this study, the 2*c* reference value was determined by taking the mean DNA content measured for nuclei that were identified as lymphocytes and if present fibroblasts. After establishing the 2*c* reference value, the histogram was scaled up to 10*c* with a fixed number of 256 bins in order to obtain standardized histograms that cover a wide range of *c* values that potentially occur in populations of (pre)cancerous nuclei.

All ICM-DNA histograms were analyzed using the MultiCycle AV computer program (Phoenix Flow Systems, San Diego, CA, USA) according to a previously developed protocol.[21] The DNA index was calculated by dividing the modal channel number of DNA aneuploid peaks by the corresponding number of the DNA diploid peak. In case of only one cell cycle, the DNA index was set at 1.00. All ICM-DNA cases were classified in 2 subclasses, based on previous published guidelines[19] as follows: DNA diploid (only one cell cycle present) and DNA aneuploid (DNA index $\geqslant$ 1.1).

Statistical analysis

Different variables were part of the analysis: clinical parameters, DNA ploidy measurement (DNA diploid or DNA aneuploid) and dysplasia score. As for this latter variable, patients were also divided in two groups: no or mild *versus* moderate or severe dysplasia.[17] Subsequently, combinations of DNA ploidy and dysplasia scores were used: DNA aneuploidy with dysplasia (moderate or severe) *versus* the rest, as well as DNA aneuploid and/or dysplasia (moderate or severe) *versus* the rest. Development of histologically proven cancer was the analytical endpoint.

To establish the association between the clinical and biomarker variables and progression into cancer, uni- and multivariate Cox regression analyses were performed and hazard ratios (HR) including its 95% confidence intervals (CI) and associated *p*-values were calculated. Graphic plots were constructed according to the Kaplan–Meier method and differences between the curves analyzed by the log-rank test. The significance of differences of