

Ploidy analysis of field cancerization and cancer development in the hamster cheek pouch carcinogenesis model

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BACKGROUND: The hamster cheek-pouch carcinogenesis model is a well-known animal system that closely mimics the development of premalignant and malignant lesions in human oral cancer. Our aim was to numerically characterize the premalignant and malignant lesions and expressions of field cancerization in this model using ploidy as the end-point.

METHODS: To study the DNA content and proliferation status of the cells in this model we assessed the Feulgen reaction and the immunohistochemical reaction for 5-bromo-2-deoxyuridine (BrdU) in different histological areas of serial tissue sections of the cheek pouches of animals injected with BrdU.

RESULTS: Ploidy values were higher in cancerized epithelia with no unusual microscopic features (NUMF), in preneoplastic and tumor areas than in control epithelia. The aneuploidy index was higher in NUMF areas than in control and differed significantly from control in preneoplastic areas and carcinoma.

CONCLUSIONS: The unexpected alteration in DNA content observed in NUMF epithelia is of great relevance as a biomarker of field cancerized areas.

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Introduction

Field cancerization is induced by carcinogens that act on a large area of tissue and cause molecular alterations that may not be expressed as clinical or histological lesions but increase the risk of malignant transformation (1).

The best known carcinogens that affect the mouth are tobacco and alcohol. They affect virtually the whole mucosa and are clearly associated with the development

of squamous cell carcinoma as single or multiple foci that originate in premalignant lesions, in areas in the vicinity of premalignant lesions or in areas with no prior observable lesion (2).

Taking histologically normal epithelia adjacent to human oral carcinomas as a model of a cancerized field, several molecular analyses have evidenced the existence of early genomic alterations (3). In these tissue areas, microsatellite alterations (4) or chromosomal instability (5) have been demonstrated. Genetically altered cells form clonal populations with growth advantage and a high proliferative rate (6, 7).

It is now widely accepted that accumulation of different genetic alterations are necessary for malignant progression. Among them, mutations affecting normal chromosome segregation may lead to abnormal DNA nuclear content or aneuploidy (8). Cells are generally called aneuploid when their DNA content does not reach the normal diploid value (2c) or, much more commonly, when they exceed the tetraploid value.

Most human malignant tumors exhibit different grades of aneuploidy that correlate with histopathological and clinical behavior. This knowledge was mainly obtained by applying ploidy analysis with cytophotometric techniques. Quantitative evaluation of nuclear DNA by cytophotometry is less sensitive than molecular cytogenetic or karyotypic analysis and does not provide information on specific chromosomal aberrations. However, as it can be easily performed on fixed histopathology specimens, it has become a useful tool in diagnosis and prognosis of solid malignant tumors in general and in oral cancer in particular (9–13). In recent years, this technique has been used to demonstrate DNA aneuploidy in leukoplakia, the most recognized precancerous lesion of oral mucosa (14–17). These data strongly point to aneuploidy as a cause rather than a consequence of malignant transformation.

We herein report cytophotometric detection of ploidy alterations in a cancerized field, even before the microscopic manifestation of any morphologic changes, using the hamster cheek pouch model of oral cancer. This widely used model closely mimics the development of premalignant and malignant lesions in the human oral

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cavity (18–22). As in human oral cancer, although the carcinogen is applied on the whole surface of the cheek pouch, the process of cancerization is not uniform. The severity of the lesions increases with time. However, at the end of the process, tumors coexist with preneoplastic lesions and areas of apparently normal mucosa. These features render the model of hamster cheek pouch oral cancer ideal to evaluate end-points that contribute to the understanding of the oral carcinogenesis process.

Materials and methods

Thirty-six Syrian hamsters (male and female), 6–7 weeks of age, 150–200 g body weight, were employed throughout. They were given food and water *ad libitum* and kept on a light–darkness cycle of 12–12 h. All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were submitted to a standard carcinogenesis protocol (20) that involves topical application of 0.5% DMBA on the right cheek pouch three times a week over a 4-month period. The control group was composed of 16 hamsters treated with vehicle (mineral oil) alone. The animals were killed at 15–16 weeks of treatment by inhalation of an overdose of sulfuric ether. Thirty minutes prior to killing the animals were given an intraperitoneal injection of a solution (5 mg/ml) of 5-bromo-2-deoxyuridine (BrdU) in saline solution at a dose of 0.08 mg BrdU/g body weight. The right cheek pouch was removed and fixed in 10% formaline. Samples that included the macroscopically evident lesions were dissected and routine processed for paraffin embedding. Hematoxylin and eosin (H&E) stained sections were employed to identify the following histological categories: carcinoma (Ca), carcinoma *in situ* (CIS), dysplasia (D), hyperplasia (H), and epithelial areas with no unusual microscopic features (NUMF). Control (C) areas were taken from cheek pouches treated with vehicle alone.

DNA cytometry

Seven micrometer sections adjacent to H&E stained sections were processed for Feulgen staining (23). Acid (5 N HCl) hydrolysis was performed for 90 min, the time interval previously identified on the hydrolysis curve for this material. The areas previously selected on the H&E sections were identified on projections of the Feulgen stained sections. Image analysis for ploidy evaluation was performed on each of these areas employing a Zeiss MPM-800 microscope with a 40× objective (NA 0.75), a condenser (NA 0.8), a Hitachi DK 7700-SX K black and white CCD camera (Hitachi Denshi Ltd., Tokyo, Japan), an image analyzer (IBAS-Kontron, Jena, Germany) and the DNA measurement software for IBAS version 1.0. For densitometric measurements, the images were stored employing a narrow band bypass filter with an absorbance peak at 560 nm. Segmentation was performed interactively. The content of DNA of lymphocytes contained in the section was taken as the diploid (2c) reference value. An additional *ad hoc* prepared software was used to correct measure-

ments for chromatic condensation of lymphocytes and section thickness (24, 25). Approximately 60 cells were measured for each selected area. The ploidy value of each nucleus was calculated as the ratio between its integrated optical density (OD) and the OD of the 2c reference value 2×. Ploidy histograms (frequency of occurrence of individual cell ploidy values) were constructed for each histological category. Aneuploidy was objectively computed on the basis of the algorithm defined by Böcking et al. (24, 26) as the 5cER (5c exceeding rate) or the aneuploidy index. 5cER is defined as the percentage of cells with a DNA content of more than 5c.

Calculation of the Ploidy value and 5cER for each region measured are incorporated in the DNA-IBAS software.

Finally, the mean ploidy and mean 5cER for each category of all cases under study were calculated.

Cell proliferation

A second adjacent 5 µm section was processed for immunohistochemical detection of BrdU to label proliferating nuclei. The histological sections mounted on silane-coated slides were dewaxed in decreasing concentrations of xylene and ethanol. They were then incubated in methanol–hydrogen peroxide (100:1) for 30 min to inhibit the activity of endogenous peroxidase. Antigen retrieval was performed in a microwave oven with citrate buffer, pH 6. Nonspecific blocking was performed by washing in 0.5 M Tris–saline buffer, 0.3% bovine albumin. The sections were incubated with the mouse antiBrdU antibody, (# 247M; Biogenex, San Ramon, CA, USA) overnight at 4°C and washed with Tris–saline buffer–albumin. The sections were incubated at room temperature for 1 h in a moist chamber with the second biotinylated antibody (dilution 1:200, antimouse Ig G; Kit Multilink, Biogenex). Finally, the sections were incubated in the same conditions with the streptavidin-peroxidase complex (Biogenex). Labeling was performed with 0.5 M Tris buffer containing 0.03% 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) and 1% hydrogen peroxide. Counterstaining was performed with hematoxylin. The sections were then dehydrated and routine mounted in balsam. As negative controls, adjacent sections were processed omitting incubation with the primary antibody.

The labeling index (LI) was calculated as the number of labeled cells over the total number of cells in the selected area (magnification: 40×), employing an image analysis software developed *ad hoc* to distinguish between positive nuclei (with DAB deposits) and hematoxylin stained nuclei. Two to five fields were evaluated for each category.

Results

Ploidy histograms showed a skew to the right from the diploid value in all the histological categories. The skew increased in relation to the severity of lesions under study. Fig. 1 shows an example of characteristic histograms. Interestingly most histologically normal epithelia

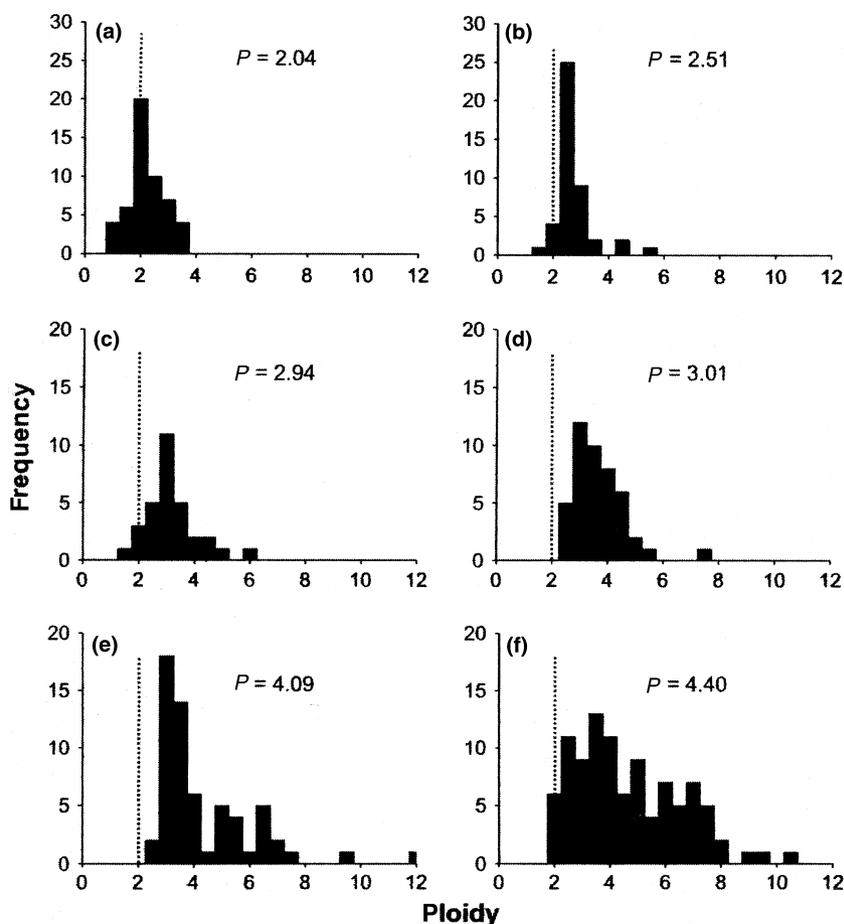


Figure 1 An example of ploidy histograms for the different histological categories: (a) control epithelium, (b) cancerized epithelium with no unusual microscopic features (NUMF), (c) hyperplasia, (d) dysplasia, (e) carcinoma *in situ*, (f) carcinoma. (b)–(f) correspond to a pouch treated with DMBA for 16 weeks. Dashed lines indicate $2c$ value. Note the skew to the right and the aneuploid events in NUMF epithelium.

(NUMF regions) evidenced a peak with a small deviation within the $2c$ – $4c$ region and some events exceeding the tetraploid value.

Mean ploidy and $5cER$ values are shown in Table 1. As expected, carcinomas and carcinomas *in situ* exhibited

Table 1. Ploidy and proliferation analysis in the different stages of carcinogenesis

Histological categories	Ploidy	$5cER$	LI	<i>n</i>
Control	2.22 ± 0.10	0.33 ± 0.51	0.04 ± 0.01	16
NUMF	2.69 ± 0.08^a	5.70 ± 1.02^a	0.10 ± 0.02^a	50
Hyperplasia	2.97 ± 0.12^a	$7.14 \pm 1.60^{a,f}$	0.08 ± 0.01	41
Dysplasia	2.93 ± 0.15^a	$7.08 \pm 1.67^{a,f}$	0.10 ± 0.02^a	21
Ca <i>in situ</i>	3.56 ± 0.33^a	$13.01 \pm 3.98^{a,b}$	0.11 ± 0.02^a	8
Carcinoma	4.35 ± 0.14^a	$12.93 \pm 1.92^{a,b,c,d,e}$	$0.17 \pm 0.02^{a,b,c,d,e}$	26

Data are expressed as mean \pm SD. *n* is the number of areas measured for each histological category. Areas were taken from 20 experimental and 16 control animals.

NUMF, cancerized epithelia with no unusual microscopic features; $5cER$, $5c$ exceeding rate (aneuploidy index); LI, labeling index of proliferation.

Duncan test post-significant ANOVA ($P \leq 0.001$). ^acompared with control; ^bcompared with NUMF; ^ccompared with hyperplasia; ^dcompared with dysplasia; ^ecompared with CIS; ^fcompared with carcinoma.

a statistically significant increase as compared to control. Mean ploidy values were near the tetraploid range. However, a considerable number of cells exhibited an aneuploid DNA content.

The results for preneoplastic areas and histologically normal areas (NUMF) were remarkable. Mean ploidy was significantly higher than for control and aneuploid cells were detected in all of these regions measured.

The cell proliferation data showed a marked increase in the labeling index in NUMF areas as compared to the control epithelia. This rate of proliferation was the same in preneoplastic lesions and in carcinomas *in situ* and reached the highest values in tumors (Table 1).

Discussion

Our results support the concept that aneuploidy is a very early event in oral carcinogenesis. When abnormal amounts of DNA reach certain levels, they can be detected by cytophotometry. This method has already been applied to the study of oral leukoplakia, and has shown an association between aneuploidy and histological dysplasia and the risk of malignant transformation (6, 14–16). The detection of aneuploidy in non-dysplastic leukoplakia has been of even greater significance (17).

Our study moves forward in that it proves the possibility of detecting aneuploid cells in a cancerized field before the appearance of histological lesions. Alterations in karyotype of hamster cheek pouch cells as early as 2 weeks after the beginning of tumor induction were reported in genetic studies (27). We found that a cytophotometrically detectable accumulation of genetic damages occurs before the premalignant lesions appear.

The great advantage of the method of quantitative evaluation of DNA by image analysis lies in the fact that it is possible to assess small areas of tissue sections, preserving tissues and cell architecture. However, it is less sensitive than the cytogenetic methods. Its sensitivity has been estimated at 5% of the normal DNA content, i.e. one to two whole chromosomes (6). The extrapolation of data from experimentally induced tumors to sporadic human cancers should be cautious. The hamster cheek pouch is certainly an excellent model to study genetic changes in the cancerization process, and particularly those involved in the field cancerization phenomenon. However, the carcinogen employed is a potent, aggressive genotoxic agent that induces complete cancerization in less than 4 weeks. Within this context, the values for hyperplastic and dysplastic lesions cannot be compared mathematically with those described for human leukoplakia.

The 5cER index is a way of measuring aneuploidy that contributes to the objectivity of routine evaluation of ploidy histograms. An index of 10, i.e. 10% of cells with DNA content in excess of 5c, is taken to represent installed malignancy. Percentages ranging between 2 and 4 should be taken to indicate an increased risk of malignant transformation (16, 24, 28, 29). The values of 5.7 ± 1.02 reported herein for NUMF areas reveal that many of these areas exhibit more than five aneuploid events. If these observations could be extrapolated to human biopsies, an increased 5cEX would suggest the existence of a cancerized field.

However, the method could produce false negatives. The failure to detect aneuploidy would not imply the absence of anomalies given that clones of transformed cells may exist in the 2c–4c range. Within this context, labeling of proliferating cells may be a useful complementary diagnostic tool given that an increase in proliferative capacity has been demonstrated to be an early event in the process of cancerization (7). The present study reports marked increases in the labeling index of bromo-deoxyuridine in NUMF regions and, as expected, in dysplastic and tumor areas. This marker is not easily applicable to human biopsies. However, other very sensitive proliferation immunohistochemical markers can be used (30).

Given the complexity of the process of malignant transformation, different end-points can contribute to a better understanding of oral carcinogenesis. Those that can be applied to material that has been routine processed for histopathological diagnosis may also have a practical significance. In a previous paper we demonstrated the efficacy of silver stained Nucleolar Organizer Regions (AgNOR) to evidence changes in histologically normal areas contiguous with human oral carcinomas

(31) and in NUMF areas of cancerized cheek pouch (32). A set of reactions that are easily applicable to sections adjacent to those stained with H&E, e.g. K67 proliferation marker, mutant P53, AgNORs and nuclear DNA quantitative evaluation, would be very effective in detecting field cancerized areas. These markers would, eventually, also be effective in monitoring the action of preventive measures such as abstention from alcohol consumption and from use of tobacco, and the administration of cancer-preventive drugs.

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