DNA cytometry of oral leukoplakia and oral lichen planus

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ABSTRACT

Background

DNA cytometry is a technique that allows densitometric analysis of nuclear DNA of cells and in this way the evaluation of DNA ploidy.

Objective

The purpose of the present study was the evaluation of DNA ploidy in oral leukoplakia and oral lichen planus and correlation with histological dysplasia.

Methods

We analyzed oral incisional biopsies from a group of 40 patients with oral leukoplakias (20 homogeneous and 20 non-homogeneous) and 45 patients with oral lichen planus (25 erosive, 20 reticular), by DNA cytomorphometry, after conventional histological examination of sections stained with haematoxylin and eosin.

Results and Conclusions

Eight of 20 non-homogeneous leukoplakias showed aneuploidy; (3 showed mild aneuploidy) and only 2 specimens of erosive lichen planus showed moderate aneuploidy, while all 20 cases of reticular lichen planus were diploid. There was not concordance between DNA ploidy and the degree of histological assessed dysplasia.

Key words: *DNA cytometry and ploidy.*

INTRODUCTION

Oral carcinoma is sometimes preceded by potentially malignant (premalignant) lesions such as leucoplakia or erythroplakia (1-3) . Up to 10% of patients with leucoplakia have invasive carcinoma in the lesion (6%) (4) and many will develop carcinoma at some time in the future –up to 30% in those with dysplasia (5) . However, there is inconsistency and disagreement in grading lesions for dysplasia (6), the behaviour of dysplastic leucoplakias, is unpredictable, and more reliable markers to predict the potential for malignancy are required. The ideal would be not only to perform a correct diagnosis but to have a reliable technique able to individualize those lesions that show great potential for cancer development, to implement treatment before (malignant transformation) (7) .

Molecular and genetic changes arise during carcinogenesis and could lend themselves to be markers of transformation. Mutations in p53, loss of heterozygosity (LOH) and chromosomal polysomy are all associated with progression to carcinoma, and may be predictive when used and analysed in combination (8-11) . Routine use of these techniques is, however, hampered by the complexity of the tests and lack of facilities in many routine laboratories (12,13) .

As a surrogate for such individual molecular markers, measurement of gross genomic damage, in the form of aberrant DNA content, could be a valuable method for prognostication of malignant and premalignant lesions (14,15) . Relativity recently, there has been a major advance in this area with the use of automated image cytometry to measure ploidy in nuclei extracted from routinely processed paraffin sections. This system may be more sensitive in the evaluation of oral potentially malignant lesions into 'low' and 'high' risk (16,17) .

In this study we have analyzed two oral potentially malignant lesions by DNA cytometry and have compared the results with the degree of histological dysplasia.

PATIENTS AND METHODS

We studied incisional biopsies from the lesions of 40 patients with oral leukoplakia (20 homogeneous and 20 non-homogeneous) and 45 patients with oral lichen planus (25 erosive, 20 reticular).

All patients consented to the research, approved by the Local Ethical Committee.

Oral biopsies were taken under local anaesthesia using an 8 mm

biopsy punch. The biopsy was fixed in formalin, embedded in paraffin and divided into two, half for histological examination for diagnostic confirmation, the other half being sectioned in sections from 6-7 microns to be evaluated by densitometry with the technique of DNA cytometry after colouration with Feulgen dye. Acid hydrolysis was carried out in 5 N HCl for 1 h at ambient temperature, with the purpose of removing purines to unmask the aldehyde groups.The aldehydes react with the Schiff reagent that is converted into its coloured form. A universal microspectrophotometer 30 (UMSP 30, Zeiss) connected to an image analyser (IBAS 2000; Kontron Electronics, Zeiss) was used for DNA cytometry.

Before proceeding to quantitative DNA analysis, the image analysis system was calibrated using cellular populations of known DNA content. Ploidy determination for every test section was performed comparing the value of integrated optic density (IOD) of 100 or 150 nuclei of every section examined with the IOD of 20 or 30 control lymphocytes (13).

The nuclear density was calculated by the analyser discriminating, on a scale of varied grey tonalities from 0 to 255, and comparing the values of ploidy of lymphocytes in the sample, with the nuclei of the pathological keratinocytes examined.

This permitted the construction of a histogram for every analyzed sample.

This is expressed by analysis of data with the following expressions:

- 2cDl = the rate of deviation from the 2c value (sign of diploidy with cell in Go phase);
- 5cEX = the percentage of nuclei that are over 5c (ploidy or aneuploidy);
- MG = the degree of malignancy- being the logarithmic transformation of 2cDl (14).

RESULTS

Eight of 20 cases of non-homogeneous leukoplakias showed an histogram with varied degrees of aneuploidy, with a 5cEX between 5,06% and 9,20% and a degree of malignancy between 0,018991 and 0,07956 (Table 1). Only 3 of the 20 sections of homogeneous leukoplakias showed a picture of mild aneuploidy with a 5cEX between 5,04% and 6,28% and a degree of malignancy between 0,064782 and 0,068754 (Table 2).

Table 1. *Numerical representation of histograms for non-homogeneous leukoplakias compared with dysplasia.*

N° patients **Non-homogeneous leukoplakia**	**2cDI**	**5cEX**	**MG**	**Dysplasia**
1	0,408021	0,00%	0,00	None
2	1,18	2,16%	0,007871	None
3	1,49	**5,46%**	**0,02445**	None
4	0,890233	2,15%	0,005726	None
5	0,405065	0,00%	0,00	None
6	1,06	**5,06%**	**0,018991**	None
7	3,37	**9,20%**	**0,07956**	**Light (OIN1)**
8	0,410101	0,00%	0,00	None
9	2,15	**6,98%**	**0,063848**	None
10	0,5050977	0,00%	0,00	None
11	0,790547	1,18%	0,005737	None
12	0,38	1,06%	0,007871	None
13	0,268752	0,00%	0,00	None
14	2,48	**7,35%**	**0,070623**	**Middle (OIN2)**
15	0,340103	0,00%	0,00	None
16	0,8412	2,05%	0,006841	None
17	0,2640931	0,00%	0,00	None
18	3,52	**9,11%**	**0,065735**	None
19	2,06	**5,06%**	**0,029974**	None
20	2,32	**9,19%**	**0,0754326**	***Middle (OIN2)***

Bartlett's statistic = 165,8
P value <0.0001

- *2cDl (index of deviation) = the rate of deviation from the 2c (c= chromosome) value (sign of diploidy with cell in Go phase);*
- *5cEX (exceeded 5c) = the percentage of nuclei that are over 5c (ploidy or aneuploidy);*
- *MG = (malignity grade) the degree of malignancy- being the logarithmic transformation of 2cDl [12].*
- *(OIN) = oral intraepithelial neoplasia*

In the lichen planus group, 2 erosive cases showed pictures of moderate aneuploidy with 5cEX between 5,25% and 6,48% and a degree of malignancy between 0,067473 and 0,068634 (Table 3), while all the cases of reticular lichen planus showed diploidy (Table 4).

We compared these data with the degrees of dysplasia on histological examination and analysed the results by Bartlett's test for equal variances but found no reliable correspondence between dysplasia and aneuploidy (Table 1, 2, 3, 4).

Patients with leukoplakia and aneuploidy have therefore been recalled and treated with a more extensive surgical excision. The patients with erosive lichen planus and aneuploidy have been treated with local radiotherapy and more frequent and careful follow up.

DISCUSSION

Experience has taught us that certain cellular and tissue alterations are associated with malignancy and premalignancy (18,19) . Altered cells appear to be more primitive than normal and so these changes are presumed to be examples of immature or inappropriate differentiation, although pathologists typically refer to them as dysplasia or atypia (20-22) . Animal models for oral carcinogenesis have reliably demonstrated that epithelium passes through stages of increasingly severe dysplasia prior to the onset of invasive neoplasia when the malignant cells visibly break through the epithelial basement membrane (18, 23) . However, the ability of any pathologist to confidently exclude the presence of invasion must surely be questionable.

Table 2. *Numerical representation of histograms for homogeneous leukoplakias compared with dysplasia.*

N° patients **Homogeneous leukoplakia**	**2cDI**	**5cEX**	**MG**	**Dysplasia**
1	0,704071	0,00%	0,00	None
2	1,20	2,06%	0,0060784	None
3	0,27459	0,00%	0,002445	None
4	1,59	**5,15%**	**0,067654**	None
5	2,46	**6,28%**	**0,068754**	**Light (OIN1)**
6	0,36476	0,00%	0,001447	None
7	0,35474	0,00%	0,004916	None
8	2,15	**5,04%**	**0,064782**	**Light (OIN1)**
9	0,25369	0,00%	0,0067548	None
10	0,7040418	0,00%	0,00	None
11	0,697567	1,13%	0,003489	None
12	0,2878	1,06%	0,005741	None
13	0,268752	0,00%	0,00	None
14	0,48256	0,00%	0,00	None
15	0,245173	0,00%	0,00	None
16	0,5457	1,05%	0,005831	None
17	0,293951	0,00%	0,00	None
18	0,62152	1,08%	0,0057345	None
19	0,67294	1,06%	0,024954	None
20	0,37452	0,00%	0,0058342	None

Bartlett's statistic =150,0
P value <0.0001

Table 3. *Numeric representation of histograms for erosive lichen planus compared with dysplasia.*

N° patients / Erosive lichen	2cDI	5cEX	MG	Dysplasia
1	0,604071	0,00%	0,00	None
2	1,40	1,16%	0,004047	None
3	0,37558	0,00%	0,004435	None
4	0,42567	0,00%	0,00	None
5	1,67	**5,25%**	**0,068634**	None
6	2,57	**6,48%**	**0,067473**	**Light (OIN1)**
7	0,6746	0,00%	0,004142	None
8	0,53464	0,00%	0,009246	None
9	0,5854	1,42%	0,0067424	None
10	0,45649	0,00%	0,0074468	None
11	0,64578	0,00%	0,00	None
12	0,496571	1,23%	0,0047839	None
13	0,37548	0,00%	0,007441	None
14	0,286742	0,00%	0,00	None
15	0,38546	0,00%	0,00	None
16	0,425713	0,00%	0,00	None
17	0,34457	1,35%	0,008741	None
18	0,492853	0,00%	0,00	None
19	0,524522	1,28%	0,004724	None
20	0,762853	1,06%	0,028753	None
21	0,47357	0,00%	0,006362	None
22	0,54817	1,15%	0,004287	None
23	0,37845	0,00%	0,00	None
24	1,30	2,20%	0,006078	None
25	0,65741	0,00%	0,004329	None

Bartlett's statistic = 195,0; P value <0.0001

Table 4. *Numeric representation of histograms for reticular lichen planus compared with dysplasia.*

N° patients / Reticular lichen planus	2cDI	5cEX	MG	Dysplasia
1	0,347169	0,00%	0,00	None
2	0,403072	0,00%	0,00	None
3	0,284571	1,06%	0,0040874	None
4	0,46472	0,00%	0,00	None
5	0,297521	0,0%	0,047456	None
6	1,06	1,28%	0,0067253	None
7	0,34474	0,00%	0,002475	None
8	0,45743	0,00%	0,002967	None
9	0,24736	0,00%	0,00	None
10	0,6034474	0,00%	0,00	None
11	0,697567	1,13%	0,003489	None
12	0,3884	0,00%	0,00	None
13	1,14	1,34%	0,0042578	None
14	0,38546	0,00%	0,0038472	None
15	0,245726	0,00%	0,00	None
16	0,27548	0,00%	0,00	None
17	0,389256	0,00%	0,0042752	None
18	0,4215	0,00%	0,0056438	None
19	0,374254	0,00%	0,00	None
20	0,247457	0,00%	0,00	None

Bartlett's statistic =120,4; P value <0.0001

As a general rule, fewer than 20% of oral leukoplakias will demonstrate dysplasia at presentation, and 8% will have severe dysplasia (19) . On the other hand, erythroplakias show severe changes more than 90% of the time (14) . However, Chiesa et al has shown that malignancy was present in around 6% of leukoplakias excised even when malignancy was unsuspected from preoperative biopsy (4) .

The clinical history of an oral cancer in 70-80% of the cases reveals a precedent precancerous lesion (leukoplakia or oral erythroplakias) (2, 18).

Specific alterations of individual epithelial cells are important in the determination of epithelial dysplasia. Cells and nuclei take on a more primitive appearance, similar to those of basal cells with enlarged nuclei (nuclear hyperplasia), dark-staining nuclei (hyperchromatism), enlarged, often eosinophilic nucleoli (prominent nucleoli), and with an increased nuclear-to-cytoplasmic ratio (19). These cells also appear to be crowded more closely together than normal keratinocytes (increased cellular density). Such changes are not exclusive to carcinogenesis, and may be seen in reactive epithelium or epithelium influenced by a variety of systemic alterations (24).

While the microscopic features of dysplasia are now relatively well established, few investigations have actually followed lesions with specific dysplastic grades or changes in order to determine their natural history. This is, of course, made quite difficult by the following facts:

• the biopsy procedure itself has removed the cells upon which the diagnosis is based;

• the more severe dysplasias are typically removed or destroyed prior to follow-up;

• the grading of dysplasias is an extremely subjective pursuit and there is often poor correlation between pathologists, even between very experienced pathologists or even within a pathologist on repeat exposure to the same specimen (25-27) .

Keeping the above caveats in mind, as a general rule it seems to be accepted that the biological behavior of severe epithelial dysplasia and carcinoma in situ are identical, or so similar as to make the distinction pointless: investigations have found that 20-35% of severely dysplastic lesions develop carcinoma, which is similar to the figures for carcinoma in situ (28) . In contrast, mild epithelial dysplasias so seldom eventuate in carcinoma, and are so similar to reactive epithelial changes, that few pathologists consider them a serious threat or recommend complete removal of the associated white lesion (29) .

The degree of dysplasia reflects the molecular and biology changes (17) .

Flow cytometry and image cytometry add significantly to the pathologist's armamentarium to assess nuclear changes associated with eventual cancer development (30) .

The analysis of potentially malignant oral lesions is the measurement of nuclear DNA content (DNA ploidy), a surrogate measure of gross genetic damage. Normally, a non-dividing somatic cell contains a diploid amount of DNA in 23 pairs or 46 chromosomes. In somatic cells, if a doubling of the DNA during S-phase occurs without a subsequent cell division, the nucleus will then contain quadruples of the DNA, making the cell tetraploid. Multiple copies of DNA in excess of diploidy is termed polyploidy. If the chromosomes are not uniformly distributed to the daughter cells, or if parts of chromosomes become detached, the chromosomal segregation during mitosis is termed unbalanced - a situation termed aneuploidy and commonly observed in many cancers (31,32) .

In this way the histological and morphometric study of individual dysplastic components in precancerous lesions can be integrated from a study of the chromosomal content of nuclei. The present study, represented by densitometry of DNA carried out on lesional biopsy, represents a usable method to determine premalignancy.

The precancerous lesions and the dysplasias show an abnormal DNA content (aneuploid DNA) in comparison to the diploidy of normal epithelium (33) . Since aneuploidy of DNA is considered to be a marker of malignancyy, it can define potentially malignant lesions (34) .

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