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DNA aneuploidy and dysplasia in oral potentially malignant disorders: Association with cigarette smoking and site

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SUMMARY

To date there are still no reliable biomarkers for oral potentially malignant disorders (PMDs) to predict the risk of progression to squamous cell carcinoma (SCC). Within a prospective clinical trial of patients with PMDs, DNA content flow cytometry (DNA FCM) was evaluated for 60 PMDs using fresh samples obtained by a dermatological curette. There were 6/42 PMDs without dysplasia, but with DNA aneuploidy, versus 8/18 with both dysplasia and aneuploidy ($p = 0.02$). When the tongue and the buccal mucosa, the two most common sites in the present series of cases were compared, dysplastic PMDs were mainly located on the tongue ($p = 0.01$). Tobacco smokers, who preferentially developed PMDs in the buccal mucosa at a younger age than non-smokers ($p = 0.002$), had fewer dysplastic PMDs than did non-smokers ($p = 0.01$). Dysplasia was significantly linked to DNA aneuploidy ($p = 0.03$) in smokers. The present data suggest that aneuploidy is an early event in oral carcinogenesis and that the influence of tobacco varies according to subsite and patient age. When DNA FCM of PMD samples are obtained by curette scraping, extensive areas can be covered with a minimally invasive, rapid, inexpensive procedure. Moreover DNA FCM of these samples appears easy amenable to routine analysis. Further research on larger numbers of PMDs should be carried out to determine whether DNA FCM plays a role in the prediction of risk of PMD transformation.

Introduction

It is generally accepted that the evolution of PMDs to SCCs takes place through dysplasia, or squamous intraepithelial neoplasia.[1,2] However, it has also been observed that "carcinoma can develop from lesions in which epithelial dysplasia has not been diagnosed in previous biopsies".[3] In practice, though no universally accepted guidelines for PMD management exist, the presence of dysplasia is often a discriminator in deciding whether or not an excision should be made.[4] Moreover, the diagnosis of dysplasia is heavily limited by the subjectivity of the operator.[4,5] Furthermore, it is doubtful whether an incisional biopsy from PMDs is reliable and representative of the histological findings of the whole lesion.[6]

Therefore, many authors have suggested that secondary prevention of SCCs from PMDs should adopt, not only the diagnosis of dysplasia, but also more objective biomarkers of the risk of transformation. Indeed the need to have molecular neoplastic progression markers in oral PMDs was voiced more than 15 years ago[7–10] and their importance has recently been confirmed.[11]

Although, the role of DNA aneuploidy in PMDs was recently discussed in a review article,[12] the relevant weight of several studies cited in this review was nullified by the fact that these studies were later retracted. On the other hand, similar studies were reported for other premalignant lesions including Barrett's esophagus,[13] ulcerative colitis,[14,15] colorectal adenomas,[16] melanocytic skin nevi[17] and cervical lesions[16] suggesting that DNA aneuploidy has an important prognostic significance.

DNA FCM was presently performed using fresh multiple samples obtained by curette scraping from wide superficial oral regions of clinically suspicious lesions. Since the length of follow-up for the patients was not long enough to obtain clinical endpoints, herein we report here the intermediary results obtained by investigating the association of DNA aneuploidy with the clinical and histopathological parameters. The present data on aneuploidy were also compared with the scanty data obtained by FCM available in the literature.

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Materials and methods

Patient data

Within the frame of a prospective study of patients affected by PMDs, DNA FCM measurements for a subgroup of 57 patients corresponding to 60 PMDs were made. Written informed consent was obtained before performing PMD curette and biopsy samplings. Patients with either oral lichen planus, or proliferative verrucous leukoplakia, were excluded from the present series. The average age of the patients were 63 for the 27 females and 59 for the 30 males. The PMD sites were as follows: tongue (25/60; 41.7%), buccal mucosa (20/60; 33.3%), floor of the mouth (7/60; 11.7%), gingiva/alveolar ridge (3/60; 5.0%), lip (3/60; 5.0%) and soft palate (2/60; 3.3%). The patients were also interviewed as to their tobacco and alcohol habits and the results were recorded. Cigarette smokers were almost all characterized by heavy smoking habit (more than 10 cigarettes per day). Former smokers (defined as those who had stopped smoking at least 6 months before the interview) were not considered in the present analyses. The consumption of at least one alcoholic unit (au) per day (1 unit = 8–10 g of ethanol = 1 glass of wine = 1/4 l of beer = 1 measure of liqueur) was considered alcohol exposure.

Sampling procedures and histology

Scrapings for FCM and tissue for morphological evaluations were obtained from each individual PMD. Scrapings from normal mucosa were also obtained from 13 healthy donors to provide tissue specific DNA diploid control cells. The surface of the oral lesion was scraped with a disposable dermatological curette (Acu-Dispo Curette®, Acuderm Inc., Ft. Lauderdale, FL, USA), as previously detailed,[18] taking care to cause slight bleeding to ensure that the basal layers of the epithelium had been collected. The scraped material was then either immediately submitted to FCM analysis or stored at +4 °C to be processed within 24 h. The histological diagnosis for the assessment of the presence of dysplasia was carried out by a specially trained pathologist, according to the WHO guidelines.[2]

Sample processing and DNA FCM

The small tissue fragments obtained by the curette were mechanically disaggregated and the cell suspensions were centrifuged at 300 rpm for 5 min to form a pellet and processed according to the Vindeløv method[19] using the Cycle Test Plus Kit (Becton Dickinson, San Josè, CA, USA). Briefly, after 10 min of trypsin digestion and treatment with RNAase and trypsin inhibitors for 10 min at room temperature, the cell suspension was stabilized and stained by adding propidium iodide and spermine. The samples were incubated at room temperature in the dark for 10 min and then filtered using a disposable Dako 30-μm Filcons filter (Dako, Glostrup, Denmark).

Nuclear DNA content FCM measurements were performed using a FACSCalibur (Becton Dickinson, San Josè, CA, USA) FCM equipped with an argon-ion laser providing a 488 nm beam and proper filters for propidium iodide excitation and collection of the red emission fluorescence. FCM measurements and DNA histogram analyses were carried out according to internationally agreed criteria.[20,21] Chicken erythrocyte nuclei (DNA QC Particles, Becton Dickinson, San Josè, CA, USA) were used to control the instrument linearity, resolution and doublet discrimination. Normal human lymphocytes were processed in parallel with the samples to be used as sex specific DNA diploid reference control cells. FCM data analysis was performed using the ModFit 5.2 computerized software program (Verity Software House, Topsham, ME). At least 20,000 cells were acquired for each sample at a speed of 20–100 cells/s. Any DNA histograms with excessive debris (>20%) or a coefficient variation (CV) of the G0/G1 peak in excess of 7% were excluded. The degree of DNA ploidy (DNA Index, DI) was evaluated as the ratio of DNA aneuploid to diploid G0–G1 peaks. DNA diploidy was for DI = 1 while DNA aneuploidy was for DI $\neq$ 1.

Statistical analysis

The association of DNA ploidy status versus histological diagnosis of dysplasia and several clinical features, including site and risk habits (tobacco and alcohol), was assessed by 2 $\times$ 2 contingency tables using the SPSS 16.0 package (Apache Software Foundation, Chicago, IL, USA). A p-value of less than, or equal to, 0.05 was considered statistically significant.

Results

Data on risk habits were available for 57 patients as to tobacco habit and for 50 on alcohol. Data on DNA ploidy status as obtained by FCM were available for 60 PMDs. Control mucosa samples from the 13 healthy donors were all DNA diploid. The average CV among all DNA diploid G0–G1 peaks was 4.5 ± 1.5.

A total of 30% (18/60) of the PMDs harbored dysplasia. DNA aneuploidy was detected in 6/42 PMDs without dysplasia (14%) and in 8/18 with dysplasia (44%).

When all the 60 PMDs were taken into consideration, a significant association was observed between the absence/presence of dysplasia and DNA ploidy status (p = 0.02; Table 1). While the non-dysplastic PMDs were strongly associated with DNA diploidy (86%), the dysplastic PMDs were DNA aneuploid in 44% of the cases.

When considering only the tongue (n = 25) and buccal mucosa (n = 20), the dysplastic PMDs were more frequent in the tongue, whereas the non-dysplastic PMDs were more frequent in the buccal mucosa (p = 0.01; Table 1). Patients were subdivided into 28 non-smokers and 29 smokers: PMDs from the non-smoker group were seen to harbor more frequently dysplasia than were the smokers (46% versus 14%; p = 0.01; Table 1). The relationship between tobacco smoking and tongue versus buccal mucosa sites was investigated (Table 2). The tongue was the most common site for PMDs in the non-smokers (17/24, 71%, p = 0.02), while the smokers had PMDs preferentially in the buccal mucosa (13/19, 68%). Age and sex were also investigated for any association between tobacco and the PMD site. The average ages of non-smokers and smokers with PMDs in the buccal mucosa were, respectively, 75.6 ± 6.5 and 55.2 ± 14 years (p = 0.002).

The association of dysplasia and DNA ploidy status for both non-smokers and smokers was also tested: a significant association was observed only among smokers where 3/4 of dysplastic cases

Table 1
Dysplasia versus DNA ploidy status (60 PMDs), site (45 PMDs) and smoking (57 patients with PMDs).

	Non-dysplastic		Dysplastic		Total
p = 0.02					
DNA diploid	36	(86%)	10	(56%)	46
DNA aneuploid	6	(14%)	8	(44%)	14
p = 0.01					
Tongue	12	(41%)	13	(81%)	25
Buccal mucosa	17	(59%)	3	(19%)	20
p = 0.01					
Non-smokers	15	(54%)	13	(46%)	28
Smokers	25	(86%)	4	(14%)	29

Table 2
Tobacco smoking versus site (43 PMDs).

$p = 0.02$	Tongue		Buccal mucosa		Total
Non-smokers	17	(71%)	7	(29%)	24
Smokers	6	(32%)	13	(68%)	19
Total	23		20		43

had DNA aneuploidy (75%; $p = 0.03$; Table 3), whilst there was a strong association between the absence of dysplasia and DNA diploid PMDs (84%). A significant association between dysplasia and DNA ploidy was also observed for both alcohol abusers and smokers ($n = 36$). There was a 20% incidence of DNA aneuploidy in the non-dysplastic PMDs and a 67% incidence for the dysplastic PMDs ($p = 0.04$; Table 4).

Discussion

The present study was conducted within the framework of a prospective clinical trial aimed at the investigation of whether DNA FCM in oral PMDs may provide a reliable biomarker of risk of malignant transformation. DNA FCM was performed using fresh multiple samples obtained by curette scraping from wide superficial regions of clinically suspicious oral PMDs.[18] Since clinical endpoints were not yet available, due to the short follow-up time, this study primarily addressed the possible links of DNA aneuploidy with dysplasia, anatomical site, tobacco and alcohol.

The combined analysis of dysplasia (absence/presence) and DNA ploidy (DNA diploidy/aneuploidy) allowed for the identification of two subgroups of PMDs of particular interest: one without dysplasia but with DNA aneuploidy (6/42; 14%) and the other with both dysplasia and aneuploidy (8/18; 44%; $p = 0.02$), which are both likely to have an higher potential of transformation. If this hypothesis is proven, this finding might indeed be of relevance in the clinical management of these patients.[22]

If we consider the studies performed on PMDs by FCM,[9,23–25] partly avoiding some of the complicated comparisons and pitfalls[24,25] when including also the data obtained by image cytometry, the incidence data of DNA aneuploidy in both the present study and literature (either from fresh/frozen or paraffin embedded material) seem to be in relatively good agreement (Table 5). To the best of our knowledge, DNA aneuploidy among PMDs without dysplasia has been reported by only Saito[9] and in our series (18.2% and 14%, respectively). These data would suggest that aneuploidy may precede dysplasia or identify a subgroup of lesions in which SCC might develop without signs of preceding dysplasia as previously reported in other studies.[3,26]

Cigarette smoking was reported to be associated with a sixfold increased risk of developing PMDs.[27] To date, as far as we are aware, the possible link of tobacco smoking and alcohol abuse with dysplasia and DNA aneuploidy has not yet been extensively studied.[28,29] Noteworthy was the finding of more dysplasia in non-smokers than in smokers ($p = 0.01$; Table 1). At first sight these data appear in contrast with previous studies, which indicated that smoking represents the most important etiological factor for the development of PMDs.[27,30,31] In order to better understand these data, we tested if the PMDs from the smoker and non-smoker groups were associated to a preferential anatomical site and/or to differences in sex and age. Taking into account only the tongue and buccal mucosa a significant association between smoking and the PMD location in the buccal mucosa was observed: 68% and 29%, respectively, among smokers and non-smokers. On the other hand the tongue was the preferential location in the non-smokers (71% versus 32%). These data are in full agreement with a previous report.[31] When compared to other series in the literature, the present study seems to report a low prevalence of lesions on the floor of the mouth and a high prevalence on buccal mucosa. The present results and data from other recent papers do not show statistically significant differences (chi-square test) as to the prevalence on the tongue, floor of the mouth and buccal mucosa.[31–33] The average age of smokers with PMDs in the buccal mucosa was significantly lower (55 years) than that of the non-smokers with PMDs in the same site (76 years). Furthermore, the average age of women who smoked was lower (50.7 years) than that of male smokers (59.6 years). These results are in agreement with the data reported by Schepman.[31] In addition, though these data await further confirmation in a larger number of patients, we did observe that, in smokers the presence of DNA aneuploidy occurred in 3 of 4 dysplastic cases (75%; $p = 0.03$; Table 3), while absence of dysplasia was strongly associated with DNA diploid PMDs (21/25; 84%). These data may identify two classes of PMD smokers: those who developed lesions at a relatively young age which are still, however, non-dysplastic and DNA diploid (PMDs which might eventually regress) and a second category including smokers who already have PMDs with both dysplasia and DNA aneuploidy.

A recent retrospective study concluded that DNA aneuploid oral dysplastic lesions have a high risk of malignant progression.[34] However, that DNA aneuploidy represents a biomarker of cancer risk in oral PMDs still remains to be proven.

A quick survey of the literature on DNA aneuploidy as a biomarker of cancer risk reveals that numerous positive correlations with clinical end-points have already been published, e.g., for Barrett's esophagus[13] and ulcerative colitis.[14,15]

DNA aneuploidy, an unbalanced and abnormal DNA content, should be regarded as a consequence of chromosomal instability (CIN), which appears to contribute to cancer genesis and progression.

Overall, the present data suggest that DNA aneuploidy in oral PMDs, as detected by FCM, is an early event in oral carcinogenesis and that the influence of tobacco varies according to both subsite and patient age.

Table 3
Dysplasia versus DNA ploidy status in 29 PMD patients with tobacco smoking habit.

$p = 0.03$	DNA diploid		DNA aneuploid		Total
Non-dysplastic	21	(84%)	4	(16%)	25
Dysplastic	1	(25%)	3	(75%)	4
Total	22		7		29

Table 4
Dysplasia versus DNA ploidy in 36 PMD patients with either alcohol or tobacco smoking habits.

$p = 0.04$	DNA diploid		DNA aneuploid		Total
Non-dysplastic	24	(80%)	6	(20%)	30
Dysplastic	2	(33%)	4	(67%)	6
Total	26		10		36

Table 5
Comparison of DNA aneuploidy incidence in PMDs as evaluated by FCM using fresh/frozen (*) or paraffin embedded material (**).

1st author/year/ref.	*N*	DNA aneuploidy			
		Displastyc PMDs		Non-displastyc PMDs	
Kahn/1992/[23]**	34	8/24	(33%)	0/10	(0.0%)
Saito/1995/[9]*	50	15/39	(38.5%)	2/11	(18.2%)
Saito/1998/[24]*	39	9/32	(38%)	0/7	(0.0%)
Seoane/1998/[25]**	31	3/31	(9.7%)		
The present study*	60	8/18	(44%)	6/42	(14%)

The presently adopted sampling technique, based on a widespread scraping of the oral region with a dermatological curette, is minimally invasive, representative of extensive lesions, rapid and easy amenable to routine analysis. The CIN status in PMDs, as measured by flow and image cytometry, which should be considered as complementary[35] and both easily leading to routine analysis, is likely to reflect those aspects of tumor biology associated with abnormal replication and segregation of sister chromatids. Whether or not cytometry is a reliable tool to assess the CIN status and predict PMD risk of progression or if it is necessary to use other more sophisticated and much more expensive techniques (e.g., microarrays for analysis of DNA aberrations or altered RNA or protein expression), remains to be confirmed.

Conflict of Interest Statement

None declared.

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