Oral cancer genesis and progression: DNA near-diploid aneuploidization and endoreduplication by high resolution flow cytometry

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Abstract. Oral potentially malignant lesions (OPMLs) with dysplasia and aneuploidy are thought to have a high risk of progression into oral squamous cell carcinomas (OSCCs). Non-dysplastic "oral distant fields" (ODFs), characterized by clinically normal appearing mucosa sited at a distance from co-existing OPMLs, and non-dysplastic OPMLs may also represent an early pre-cancerous state. ODFs, OPMLs without and with dysplasia and OSCCs were investigated by high resolution DNA content flow cytometry (FCM). ODFs and OPMLs without dysplasia were DNA aneuploid respectively in 7/82 (8.5%) and 25/109 (23%) cases. "True normal oral mucosa" and human lymphocytes from healthy donors were DNA diploid in all cases and were used as sex specific DNA diploid controls. Dysplastic OPMLs and OSCCs were DNA aneuploid in 12/26 (46%) and 12/13 (92%) cases. The DNA aneuploid sublines were characterized by the DNA Index ($DI \neq 1$). Aneuploid sublines in ODFs and in non-dysplastic and dysplastic OPMLs were near-diploid ($DI < 1.4$) respectively in all, 2/3 and 1/3 of the cases. DNA aneuploid OSCCs, instead, were characterized prevalently by multiple aneuploid sublines (67%), which were commonly (57%) high-aneuploid ($DI \geqslant 1.4$). DNA near-diploid aneuploid sublines in ODFs and OPMLs appear as early events of the oral carcinogenesis in agreement with the concept of field effect. Near-diploid aneuploidization is likely to reflect mechanisms of loss of symmetry in the chromosome mitotic division. High DNA aneuploid and multiple sublines in OPMLs with dysplasia and OSCCs suggest, instead, mechanisms of "endoreduplication" of diploid and near-diploid aneuploid cells and chromosomal loss. High resolution DNA FCM seems to enable the separation of subsequent progression steps of the oral carcinogenesis.

Keywords: Oral field effect carcinogenesis, oral potentially malignant lesions, oral squamous cell carcinomas, DNA aneuploidy, flow cytometry

1. Introduction

Chromosomal instability (CIN) contributes together with gene mutations and epigenetic aberrations to cancer genesis and progression [11,20,25,28,47,51,53]. CIN was associated to the mitotic checkpoint [20, 28] and to aberrant centrosome function [6]. The mi-

totic checkpoint, in particular, monitors microtubule attachment at kinetochores during mitosis and prevents cells with unaligned chromosomes from proceeding to anaphase by inhibiting the anaphase-promoting complex/cyclosome. These CIN mechanisms can be leading to an imbalanced DNA content (DNA aneuploidy) in a cell and generate DNA aneuploid sublines, which may acquire a proliferative advantage with respect to the normal cells. DNA FCM was often adopted as a useful technique for detecting the presence of DNA aneuploid sublines in several human predisposing and preneoplastic lesions such as Barrett's esophagus [35], ulcerative colitis [33,36], colorectal adenomas [10] and oral lesions [19,31,38–41]. The FCM data provided so far for the human oral precancerous lesions were mainly derived from paraffin-embedded material of dysplastic oral potentially malignant lesions (OPMLs). In order to better investigate early oral fields of carcinogenesis and to separate them from later progression steps, we have presently included the analysis of non-dysplastic "oral clinically normal appearing mucosa sited in OPML distant fields" (ODFs; $n = 82$). In addition, we have analyzed OPMLs, which could be clinically identified as white or red lesions of the oral mucosa (leukoplakias and erythroplakias) but were classified without dysplasia at histology ($n = 109$). Two further groups analyzed were dysplastic OPMLs ($n = 26$) and oral squamous cell carcinomas (OSCCs; $n = 13$). In the present series, all samples were only from fresh/frozen material and nuclei suspensions were prepared and stained with DAPI according to an optimized protocol (see Section 2). The DNA FCM measurements were then based on the use of a dedicated instrument using UV incident light [44]. This procedure has allowed to measure G0–G1 DNA diploid control nuclei with CV values commonly near 1% and to detect DNA at high resolution for near-diploid aneuploid sublines with slight DNA changes above/below the DNA diploid control, as for example a DNA increase in a OPML of 2.4%.

2. Patients and methods

2.1. Patients

Hundred patients (57 males and 43 females, with a median age of 60 years, range 26–87) affected by OPMLs (homogeneous and non-homogeneous leukoplakias, erythroplakias and erythroleukoplakias) in single or multiple oral cavity subsites were included in this study for a total number of 135 OPMLs. Lesions were considered to be multiple when their development subsites for any single patient were different. Patients with lichen planus and proliferative verrucous leukoplakia lesions were excluded on the base of standard clinical protocols. Seventeen additional individuals without oral lesions, young non-smoker individuals who underwent surgery for the extraction of wisdom teeth (6 males and 11 females), were considered as donors of "true normal oral mucosa". The study also included 12 patients with OSCCs (9 males and 3 females, with a median age of 69 years, range 36–83). One single patient had two separate OSCCs in two different subsites. Patients were afferent to either one of these three different medical Institutions: The Oral Medicine and Oral Oncology Section of the University of Turin, The Department of Otolaryngology, "S. Martino Hospital" in Genoa and the National Institute for Cancer Research in Genoa. Patient written consent was obtained in every case according to the Institutional Ethic Committees.

2.2. Sampling procedures and histological assessment

This study included 135 OPMLs, 109 without and 26 with dysplasia, and 13 OSCCs. In addition, we investigated 82 ODFs and 17 "true normal oral mucosa" from healthy donors. Samples were obtained from the following oral anatomical subsites: the tongue, the buccal mucosa (i.e., the mucous membrane covering the inner surface of the cheeks and lips ending above and below a transition to the gingiva and including the retromolar trigone) and the floor of the mouth. An additional relatively small oral subgroup included the gingiva/alveolar ridge, the lip, the soft and hard palate.

The sampling protocol was based on the use of a disposable dermatological curette (Acu-Dispo Curette®, Acuderm Inc: Ft. Lauderdale, FL, USA) as previously detailed [24]. This protocol was adopted for all the clinically visible lesions, from the smallest to the largest ones, and also for the "clinically normal appearing mucosa". Slight bleeding was required to assure that cells were collected from both the superficial and basal layers of the epithelium. Micro-histological diagnosis from tissue fragments collected by the curette was done as previously reported [24]. In presence of relatively large lesions, punch biopses with a diameter of 3–6 mm were also collected in addition and subdivided for histology and FCM analysis. These last samples were composed of both epithelial and connective tissue through the basal layer. Fresh samples

for DNA FCM were either immediately processed or stored at $-20°C$ for later analysis. Conversely, fixed samples served for an approximate counting of the epithelial component and for the histological diagnosis. The presence of dysplasia or carcinoma on the haematoxylin–eosin stained slides was assessed by two pathologists, according to the WHO guidelines [2]. In case of disagreement, the slides were re-evaluated until a consensus was reached. As for the tumor samples of OSCCs, they contained at least 30–40% tumor cells as visually assessed on a nearby section by microscopy.

2.3. Sample processing for DNA FCM

Tissue fragments were minced on Petri dishes using scalpels and collected in 2 ml detergent solution (0.1 M citric acid, 0.5% Tween-20) [27] and then submitted to mechanical disaggregation in a disposable 50 μm Medicon using a Medimachine (DAKO, Copenhagen, Denmark). Nuclei suspensions were obtained and filtered over a 50 μm nylon sieve (CellTrics, Partec GmbH, Muenster, Germany). An absolute count of the nuclei in suspension was performed by FCM (CyFlow® ML, Partec GmbH) after 1–10 dilution in water. The final volume was calculated to obtain the concentration of 600,000 nuclei/ml. One volume (1/7 of the final volume) of detergent solution was first added followed by 10 min incubation and gentle shaking. Finally, 6 volumes (6/7 of the final volume) of staining solution (0.4 M Na_2HPO_4, 5 μM DAPI in water) were added. Samples were kept on dark for a minimum of 15 min incubation before filtering and FCM analysis. Excitation of DAPI was provided with an UV mercury lamp (HBO-100 W, Partec GmbH) and the emitted blue fluorescence was collected using a 435 nm long-pass filter. Measurements by DNA FCM, quality controls and DNA content histogram analysis were performed according to consensus criteria [9,15, 26,43]. Only samples with at least 2 separate G0–G1 peaks were considered DNA aneuploid. Sex specific human lymphocytes and "true oral normal mucosa" from healthy donors were used as DNA diploid controls. DNA Index (DI) values were evaluated as the ratio of the mean channel number of the DNA aneuploid G0–G1 peak to the mean channel number of the diploid G0–G1 peak. Thus, DNA diploid and aneuploid sublines have values respectively $DI = 1$ and $DI \neq 1$. The CV values of the G0–G1 peaks for the DNA diploid mucosa samples were used as a measure of accuracy (DNA resolution): mean CV was $1.9 \pm 0.5\%$ when obtained by a Gaussian curve fitting (FloMax Software

3.0b4 2001, Partec GmbH) and $1.5 \pm 0.6\%$ by dividing the peak width at half maximum (in channel number) by the peak mean channel and the factor 2.35. Mean CV values by the two methods using human lymphocytes were respectively $1.2 \pm 0.2\%$ and $0.9 \pm 0.2\%$.

2.4. Statistical analysis

Data collection, management and analyses were done using Microsoft Office Excel and the SPSS 16.0 software package (Apache Software Foundation, Chicago, IL, USA). The association among two variables in 2×2 contingency tables was evaluated with the Fisher exact test. A p-value $\leqslant 0.05$ was taken as statistically significant.

3. Results

Several examples of DNA content high resolution FCM measurements using DAPI stained nuclei suspensions for 4 different oral lesions with the presence of DNA near-diploid aneuploid sublines are shown in Fig. 1a and b. Sex specific nuclei suspensions from "true normal oral mucosa" and/or lymphocytes from young healthy females/males, were always characterized by a single G0–G1 Gaussian peak distribution (Table 1) and were used as DNA diploid controls ($DI = 1.0$). The corresponding CV values of the peaks were commonly near 1% and the FCM linearity was optimal (see DNA histograms in panels A_1, B_1, C_1 and D_1 of Fig. 1a and b). These low CV values and the detection of a near-diploid aneuploid subline with a $DI = 1.024$ (i.e., with a relative 2.4% increase of DNA content above the DNA control nuclei; Fig. 1a, panels B, B1 and B2) are indicative that the entire process of sample preparation and measurement provided DNA histograms at relatively high resolution. Figure 1a and b illustrates the detection among 4 oral lesions of 5 visibly well separated G0–G1 DNA near-diploid aneuploid sublines with respectively DI values of 0.95, 1.024, 1.04 and 1.12 and 1.08. These DI values could be clearly evaluated after measuring a mixed sample of lesion and control nuclei (panels A_2, B_2, C_2 and D_2), which produced a relative increase of the DNA diploid control peak-height and a decrease of the DNA aneuploid lesion peak-height (see also Fig. 1 legend).

Figure 1c (panels E, F, G and H) shows 4 additional examples of DNA histograms from oral lesions with DNA high-aneuploid sublines, respectively, with DI values 1.67, 1.72, 1.83 and 2.04. The CV values of

Table 1

Presence of single and multiple DNA aneuploid sublines among 5 different groups of oral mucosa/lesions

Oral mucosa/lesion groups	N. cases within groups	N. DNA aneuploid cases	One DNA aneuploid subline	Two or more DNA aneuploid sublines
"True normal mucosa" from healthy donors	17	0 (0%)	–	–
ODFs	82	7 (8.5%)	7 (100%)	0 (0%)
OPMLs without dysplasia	109	25 (23%)	22 (88%)	3 (12%)
OPMLs with dysplasia	26	12 (46%)	10 (83%)	2 (17%)
OSCCs	13	12 (92%)	4 (33%)	8 (67%)

Notes: ODFs: Non-dysplastic "oral distant fields" (ODFs), characterized by clinically normal appearing mucosa sited at a distance from co-existing OPMLs; OPMLs – oral potentially malignant lesions; OSCCs – oral squamous cell carcinomas. The total number of cases was 247. DNA aneuploid cases were further subdivided between those having single or multiple DNA aneuploid sublines (last two columns).

the G0–G1 DNA aneuploid peaks were always significantly larger than those of the G0–G1 DNA diploid peaks, reflecting a higher degree of chromosomal instability and loss for these high aneuploid sublines (see Fig. 1 legend). These high aneuploid sublines may either derive from DNA tetraploid cells/sublines (originated in a diploid–tetraploid "endoreduplication" jump) by extensive loss of chromosomes or from the "endoreduplication" of near-diploid cells/sublines (in particular, putative hypodiploid ones) having approximately $1/2$ DI value with respect to the high-aneuploid DI values (see Fig. 1 legend and Section 4).

Figure 2 shows three examples of "endoreduplication" of diploid/near–diploid cells/sublines (respectively, with $DIs = 1$, 1.12 and 1.08) into a near-tetraploid peak with $DI = 1.97$ (panel A), and two hypertetraploid peaks with $DIs = 2.18$ (panel B) and $DI = 2.16$ (panels C1 and C2), where the last 2 DNA histograms were obtained from 2 separate samples taken in different regions of the same oral lesion.

Table 1 reports the prevalence of DNA aneuploidy for 5 subgroups of oral mucosa/lesions: "true normal mucosa" from healthy donors ($n = 17$), non-dysplastic ODFs ($n = 82$), OPMLs without ($n = 109$) and with ($n = 26$) dysplasia and OSCCs ($n = 13$). All "true normal oral mucosa" samples were DNA diploid. The next 4 subgroups showed, instead, at least one DNA aneuploid subline in, respectively, 7 (8.5%), 25 (23%), 12 (46%) and 12 (92%) cases. Two or more DNA aneuploid sublines were detected in none of the ODFs, in 3 (12%) of the OPMLs without dysplasia, in 2 (17%) of the OPMLs with dysplasia and in 8 (67%) among the OSCCs.

All the DI aneuploid sublines ($n = 70$) were subdivided in 2 classes (Table 2): DNA near-diploid ($DI \neq 1$ and <1.4) and DNA high aneuploid ($DI \geqslant 1.4$). ODFs and OPMLs without dysplasia were characterized by near-diploid sublines respectively in 7/7 (100%) and in 21/28 (75%) of the cases. In contrast, OPMLs with dysplasia and OSCCs had high aneuploid sublines respectively in 10 out of 14 (71%) and in 12 out of 21 (57%) of the cases. The prevalence of high aneuploidy in OPMLs with dysplasia was statistically significantly higher than in OPMLs without dysplasia ($p = 0.007$). All the DI aneuploid values were graphically subdivided in smaller contiguous classes (Fig. 3): hypo-diploid ($DI < 1$), hyper-diploid in the near-diploid region ($1 < DI < 1.4$), near-triploid ($1.4 \leqslant DI \leqslant 1.6$), hypo-tetraploid ($1.6 < DI \leqslant 1.97$), tetraploid ($1.97 < DI < 2.03$) and hyper-tetraploid ($DI \geqslant 2.03$). In the present series of oral mucosa/lesions, the DNA aneuploid sublines among the ODFs were all near-diploid hyper-diploid. Both DNA near-diploid hypo- and hyper-diploid sublines were, instead, predominant among the OPMLs without dysplasia. On the contrary, high aneuploid sublines (mainly, hypo-tetraploid) were predominant among OPMLs with dysplasia and OSCCs. DNA aneuploid OSCCs were, in particular, characterized by multiple sublines (in 8 of 12 cases), which were mainly falling in the near-diploid and hypo/hyper-tetraploid regions (see also Figs 1c and 2).

4. Discussion

OSCCs appear to arise in precancerous fields defined as mucosa with cancer-related genetic alterations, which may or may not be recognized at the macroscopic clinical [3,4,46,50] and microscopic histological levels [2,18,21,30,34,52]. In the present study, we newly demonstrated that, while "true normal oral mucosa" and human lymphocytes of healthy donors were DNA diploid in all cases, non-dysplastic "clinically normal appearing mucosa fields of the oral cavity" (ODFs) in patients with OPMLs already con-
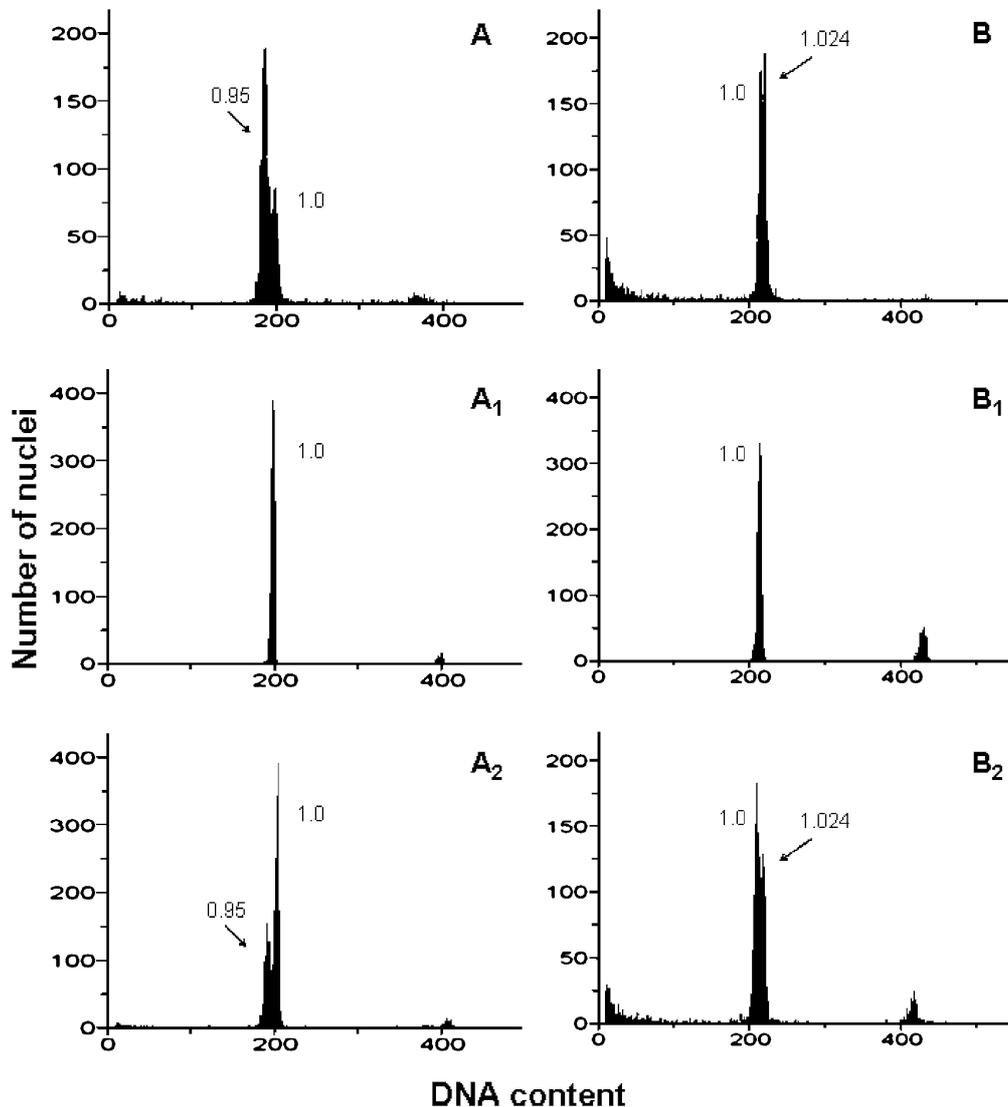
Fig. 1a. Examples of DNA content histograms from fresh/frozen oral mucosa/lesions as obtained by high resolution DNA FCM, which demonstrate the presence of DNA near-diploid aneuploid sublines (a)–(b) and high-aneuploid sublines (c). All FCM DNA content measurements were relative to internal normal diploid cells and/or sex specific externally added normal diploid cells. The calculation of the lesion DI values was therefore independent from the channel number position of the DNA diploid G0–G1 peak (near 200). During the time course of a set of FCM measurements, slight drifts of this peak could be observed from the initial position due to slight hydrodynamic and UV-lamp illumination changes. (A) Two G0–G1 peaks are visible at the channels 186 and 198; background formed by nuclei fragments is visible to the left of the peaks; S and G2/M nuclei are to the right. (A_1) Sex specific human lymphocytes of an healthy donor, used as DNA diploid control ($DI = 1$), show a single G0–G1 peak at channel 198; CV was 0.93% by fitting and 0.86% by use of the width at $1/2$ height peak formula (see Section 2); the comparably small CV values obtained for the DNA controls (A_1, B_1, C_1, D_1) indicate that the DNA content FCM measurements were performed at high resolution; in addition, the small peaks of G0–G1 lymphocyte doublets at channels near 400 define a ratio to the diploid peak of exactly 2.0 as to be expected for an optimal linearity of the measuring system. Mixing at about 50% each of the oral lesion nuclei with the DNA diploid lymphocyte nuclei (respectively, A and A_1) produced the DNA histogram shown in A_2. Comparison of the two clear-cut visible G0–G1 peaks in A and A_2 indicated that mixing produced a relative increase of the diploid peak-height (to the right) and a decrease of the other near-diploid (hypo-diploid) peak-height (to the left); one may conclude that the original cheek OPML contained a $DI = 0.95$ hypo-diploid subline characterized by a 5% DNA content decrease relative to a sex specific diploid control. B and B_2 show, similarly, two G0–G1 DNA near-diploid sublines from a tongue OPML. Following exactly the same analysis as detailed for the previous case, one ends-up with the interpretation that the original OPML contains a near-diploid hyper-diploid DNA subline with $DI = 1.024$ (i.e., with a 2.4% DNA content increase with respect to the sex specific DNA diploid control).
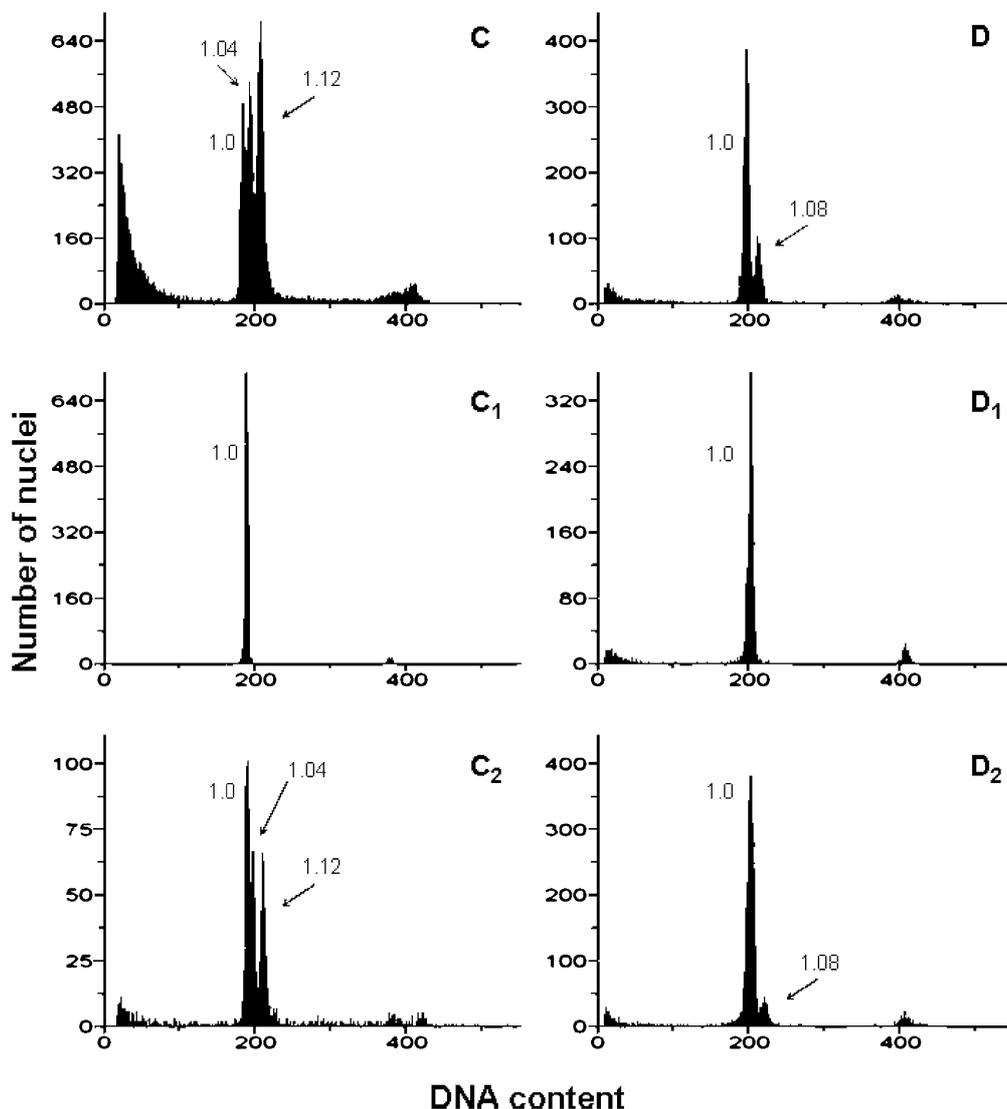
Fig. 1b. (C, C_1 and C_2) Two DNA near-diploid aneuploid sublines with DIs, respectively, 1.04 and 1.12 were detected within a gingiva OSCC. (D, D_1 and D_2) Example of a relatively small DNA near-diploid aneuploid subline with $DI = 1.08$ within a non-dysplastic soft palate OPML.

tained DNA aneuploid sublines in a subgroup of cases (7/82, 8.5%). Moreover, we demonstrated that OPMLs that could be clinically identified as white or red lesions of the oral mucosa (leukoplakias and erythroplakias), but were classified without dysplasia at histology ($n = 109$), contained already DNA aneuploid sublines in 23% of the cases. These data appear in agreement with the concept of field effect in oral carcinogenesis [3,4,46,50]. The data obtained for the non-dysplastic OPMLs, in particular, were in agreement with two previous literature reports including one from our group using an independent patient population [31, 40]. These data were, however, in contrast with other

studies, which did not detect DNA aneuploid sublines in such lesions [19,38].

The incidence of DNA aneuploidy by FCM reported in the literature ranges from about 10–40% for dysplastic OPMLs [19,31,38–41] and from about 60–80% for OSCCs [12,13]. These values may strongly depend from material type (paraffin embedded or fresh–frozen) and DNA FCM resolution. In the present study, we have performed FCM measurements at optimized conditions (fresh–frozen material, DAPI staining in nuclei suspensions, UV incident light, the use of a dedicated instrument). Correspondingly, the CV values of the G0–G1 peaks of human normal control diploid nu-
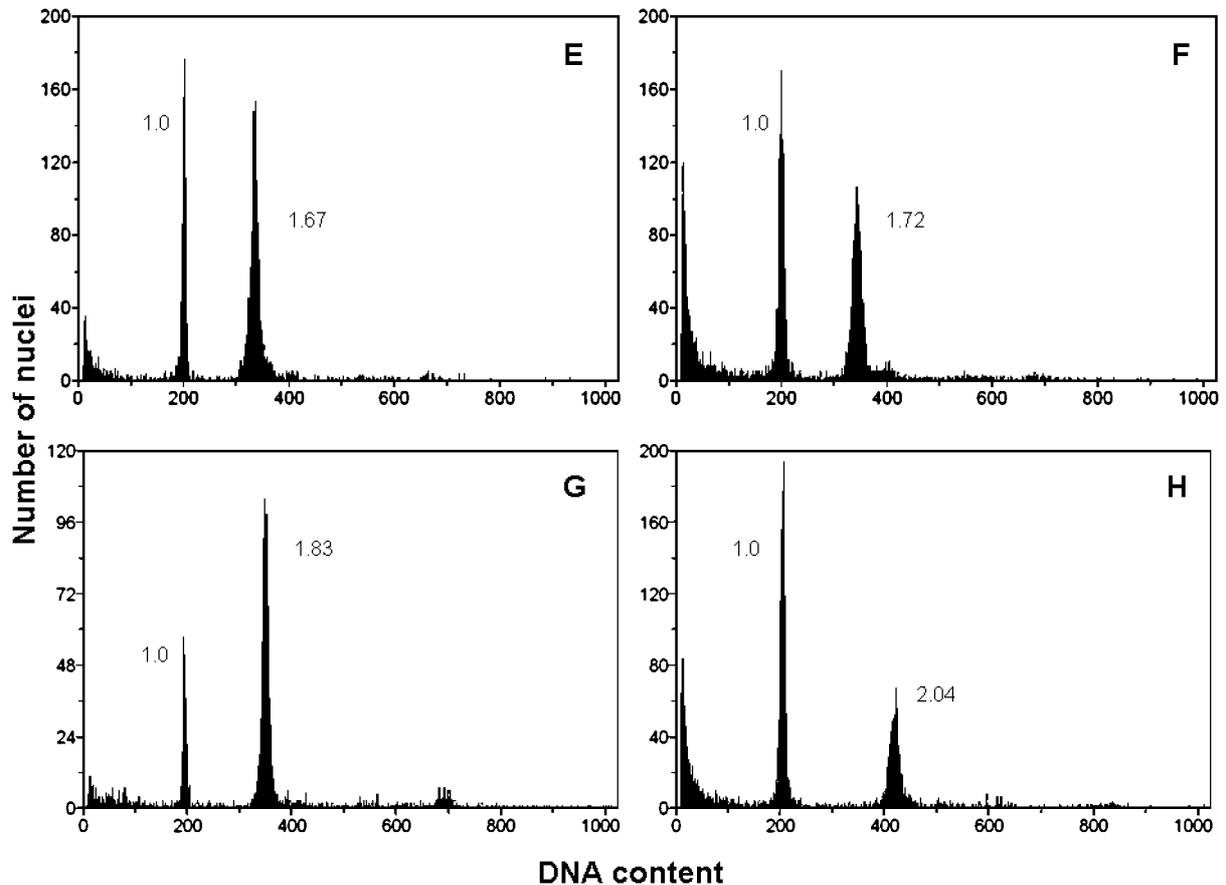
Fig. 1c. (E, F, G and H) Examples of oral lesions with DNA high aneuploid sublines ((E) tongue OPML; (F, G, H) cheek OPMLs). Mean CVs for the G0–G1 diploid and aneuploid peaks were, respectively, 1.56% and 1.90% (CVs evaluated according to the width at 1/2 height peak formula; see Section 2).

clei were commonly near 1%, while a DNA change of 2.4% was detected (see details in Sections 2 and 3). It is likely that DNA FCM at lower resolution and higher CV values would not allow to separate DNA near-diploid aneuploid sublines with only slight DNA changes above/below DNA diploidy.

The present study has additionally highlighted that ODFs and OPMLs without dysplasia were characterized by single near-diploid aneuploid sublines (Fig. 1a and b; Table 2) and that, on the contrary, OPMLs with dysplasia commonly (71%) contained high aneuploid sublines ($DI \geqslant 1.4$). High aneuploid sublines were also predominant (57%) for the OSCCs, which were, in addition, characterized by the presence of multiple DNA aneuploid sublines in 67% of the aneuploid cases. These data support a previous model of aneuploidy genesis and evolution [10]. Accordingly, a transition from DNA diploidy to near-diploid aneuploidy would be an early step of the natural history of OPMLs, while high aneuploidy would occur

as a later event in OPMLs with dysplasia and OSCCs. Chromosomal and sub-chromosomal losses and gains in oral pre-malignant lesions [4,7,22,29,37,45] might well cause DNA content changes in the near-diploid range that may be detectable by our high-resolution FCM approach. Evidence that high aneuploid values in the hyper-tetraploid region could derive by endoreduplication of near-diploid hyper-diploid sublines with 1/2 DIs was illustrated in Fig. 2B and C. A diploid–tetraploid jump [42] could also be an alternative way to generate an hypotetraploid subline (Fig. 2A). Evidence that hypodiploid cells/sublines could generate and co-exist with hypotetraploid sublines was previously reported while studying the colorectal adenoma-carcinoma transition [5,10]. These DI transitions are likely to reflect CIN and specific aspects of tumor biology [1,8,10,17,20,28]. At present, however, the mechanisms that may explain the survival, the proliferative advantage and the possible inhibition of apoptosis of these newly formed aneuploid sublines have
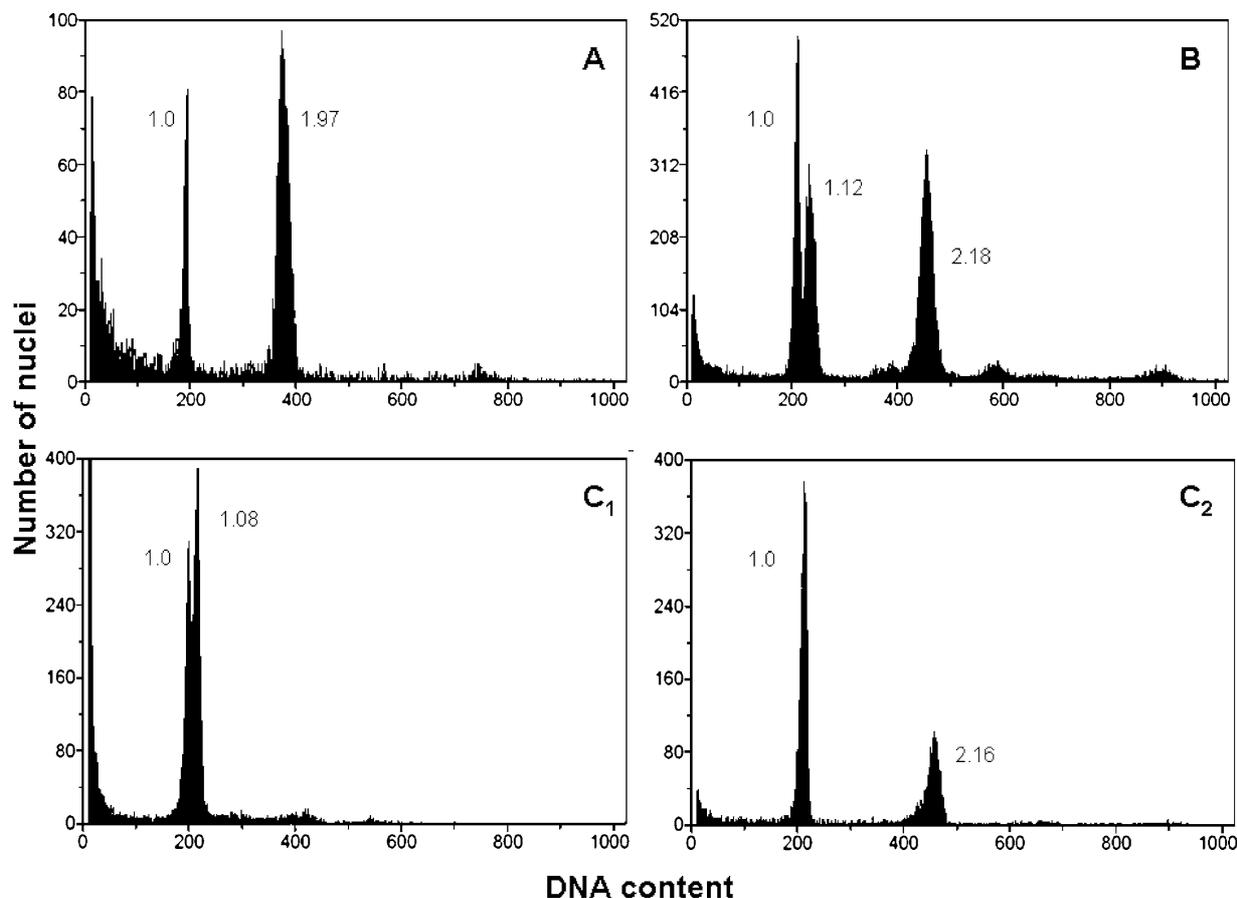
Fig. 2. Examples of "DNA endoreduplication". (A) shows a diploid/near-diploid subline ($DI = 1.0$) and a near-tetraploid subline with $DI = 1.97$. The diploid–tetraploid theory [42] proposes that a diploid cell in precursor lesions of solid tumors generates an early intermediate tetraploid state ($DI = 2.0$) and a later cascade of DNA high aneuploid sublines with lower DI values by chromosomal loss. An alternative model proposes that an early event of the natural history of the tumor precursor lesions is the generation of near-diploid aneuploid cells/sublines, which may later "endoreduplicate" into unstable high aneuploid sublines [10]. This model is illustrated (B), where a subline with $DI = 1.12$ is shown to co-exist with a second one with about twice the near-diploid DI value ($DI = 2.18$). An additional example, in which a subline with $DI = 1.08$ "endoreduplicated" into a second one with $DI = 2.16$, is reported in the panels C_1 and C_2 (C_1 and C_2 histograms were obtained from two separate samples from the same oral lesion; see also text).

Table 2

Presence of DNA near-diploid and high aneuploid sublines among 4 different groups of oral mucosa/lesions

Oral mucosa/lesion groups	N. DNA aneuploid sublines	N. DNA near-diploid aneuploid sublines ($DI \neq 1$ and $DI < 1.4$)	N. DNA high-aneuploid sublines ($DI \geqslant 1.4$)
ODFs	7	7 (100%)	0 (0%)
OPMLs without dysplasia	28	21 (75%)	7 (25%)
OPMLs with dysplasia	14	4 (29%)	10 (71%)
OSCCs	21	9 (43%)	12 (57%)

Notes: ODFs – Non-dysplastic "oral distant fields" (ODFs), characterized by clinically normal appearing mucosa sited at a distance from co-existing OPMLs; OPMLs – oral potentially malignant lesions; OSCCs – oral squamous cell carcinomas. The total number of DNA aneuploid sublines was 70.

been only partly understood [11,14,20,25,28,32,47,48, 51,53]. Additional mechanisms could be based on the normal gene dosage unbalance that aneuploidy may generate with consequent loss of growth-suppressing
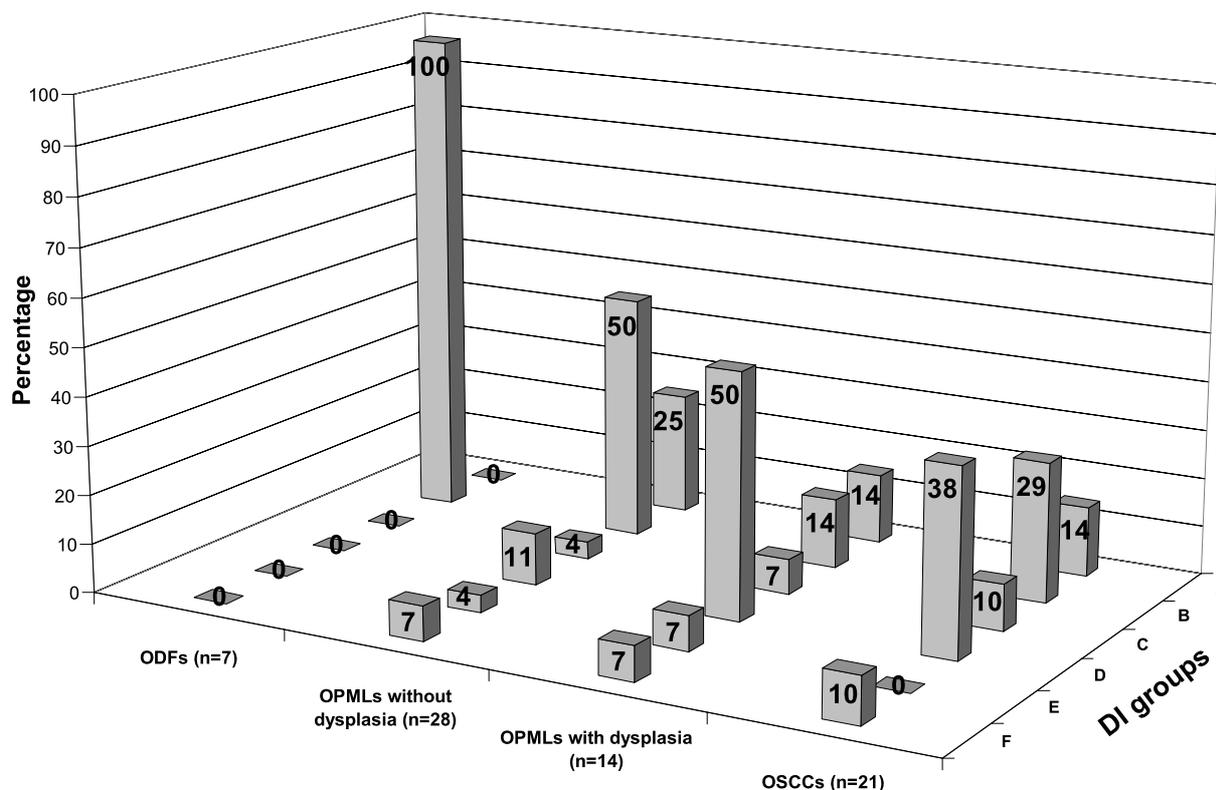
Fig. 3. DNA aneuploid sublines ($n = 70$) detected by high resolution DNA FCM in the following histological groups: non-dysplastic ODFs, OPMLs without and with dysplasia and OSCCs. The number of the corresponding DI aneuploid values were, respectively, 7, 28, 14 and 21. DI values were subdivided in the following 6 groups: (A) hypo-diploid ($DI < 1$); (B) hyper-diploid near-diploid ($1 < DI < 1.4$); (C) near-triploid ($1.4 \leqslant DI \leqslant 1.6$); (D) hypo-tetraploid ($1.6 < DI \leqslant 1.97$); (E) tetraploid ($1.97 < DI < 2.03$); (F) hyper-tetraploid ($DI \geqslant 2.03$).

genes and gain of growth-promoting genes. Chromosomal aberrations may also generate fusion genes that may activate growth-promoting genes and epigenetic events related to a selective proliferation advantage. The power of high resolution oligonucleotide-array comparative genomic hybridization and array-gene expression techniques, in combination with FCM cell by cell analysis and sorting to better approach the problem of tumor cell heterogeneity, appears particularly suitable to investigate these mechanisms and eventually lead to more refined clinical applications. In absence of this more refined information and deeper comprehension of the mechanisms, high resolution DNA FCM may help to indicate with a simple and inexpensive routine approach some of the progressive steps of a dynamic field effect process of oral carcinogenesis. Associated biomarkers may reflect the variable clinical outcomes of OPMLs, which may persist unchanged, shrink in size and even disappear, enlarge and ultimately give rise to OSCCs [23]. This expectation would be on line with several recent FCM studies investigating predisposing and preneoplastic lesions in different gastrointestinal and oral sites [16,22,29,33, 35–37,49].

Conflict of interest statement

The authors declare no conflict of interest. The Research Agreement with Philip Morris International, in particular, clearly states that "PMI shall have no right to approve any publications" and that the authors "shall have the right to publish any results".

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