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Research Article

Chromosomal Instability, DNA Index, Dysplasia, and Subsite in Oral Premalignancy as Intermediate Endpoints of Risk of Cancer

Walter Giaretti[1], Stefano Monteghirfo[1], Monica Pentenero[2], Sergio Gandolfo[2], Davide Malacarne[1], and Patrizio Castagnola[1]

Abstract

Background: Chromosomal instability and aneuploidy may represent biomarkers of oral exposure to damaging agents and early signs of clinical disease according to the theory of "oral field cancerization."

Methods: The hypothesis was tested that the DNA index (DI) values, obtained by high-resolution DNA flow cytometry (DNA-FCM), may potentially contribute to oral cancer risk prediction. For this purpose, the DI of oral fields of normal-appearing mucosa and oral potentially malignant disorders (OPMDs) in 165 consecutive patients was tested for association with dysplasia and/or the oral subsites of tongue and floor of the mouth taken as high-risk intermediate endpoints surrogate of cancer clinical endpoints. The association was evaluated by logistic regression using patient gender, age, tobacco, cigarette smoking habit, and alcohol abuse as confounding variables.

Results: Different DI models provided evidence of statistical significant associations. Subdividing the DI values in diploid, near-diploid aneuploid, and high or multiple aneuploid from both OPMDs and oral normal-appearing mucosa, ORs, respectively, of 1, 4.3 ($P = 0.001$), and 18.4 ($P < 0.0005$) were obtained.

Conclusion: Routine DI analysis by high-resolution DNA-FCM seems potentially useful to complement dysplasia and subsite analysis for assessment of oral cancer risk prediction and for a better management of the patients with OPMDs. Work is in progress to validate the present findings in a prospective study with clinical endpoints.

Impact: Identifying DNA abnormalities in oral premalignancy may lead to biomarkers of oral exposure and cancer risk and potentially to more effective prevention measures. *Cancer Epidemiol Biomarkers Prev; 22(6); 1133–41. ©2013 AACR.*

Introduction

Oral cancer, the most common neoplasm of the head and neck, accounts for about 270,000 new cases annually worldwide. The disease causes great morbidity, and the 5-year survival rate of less than 50% has not improved in more than 2 decades (1).

The natural history of oral cancer genesis and progression is characterized by the theory of "field cancerization." This theory implies the presence of carcinogens acting on a large tissue area of the mouth and the induction of genetic and genomic aberrations without histologic alterations but increasing the risk of cancer development (2–6). The contribution of carcinogens from tobacco smoking to oral cancer is well documented by epidemiologic and experimental studies (7–9).

Genetic, epigenetic, and genomic aberrations in oral normal-appearing mucosa fields (ONAMFs), oral potentially malignant disorders (OPMDs; mostly leukoplakias), and oral squamous cell carcinomas (OSCCs) have been the subject of extensive studies and reviews (3, 10–16). The potential of these aberrations to represent risk biomarkers for OPMD progression still remains unproven. The challenge of a recent study, using loss of heterozygosity profiles to analyze a cohort of 296 patients with mild/moderate oral dysplasia (17), was very relevant because it allowed stratifying oral premalignancy patients into low- and high-risk categories for progression into OSCCs. A similar role for DNA aneuploidy in OPMDs was presented in several highly ranked journals, but the relevant weight of these studies was nullified by the fact that they were later retracted (18). The relevance of DNA aneuploidy, however, was suggested for different predisposing and premalignant lesions including Barrett's esophagus (19), ulcerative colitis (20, 21), colorectal adenomas (22), and melanocytic skin nevi (23). These studies, along with other recent studies that have provided a

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deeper understanding of the chromosomal instability (CIN) and its link with aneuploidy (24–30), seem promising.

Aneuploidy indicates either an unbalanced number of chromosomes (whole-chromosome aneuploidy) or unbalanced chromosomal regions due to deletions, duplications, amplifications, or translocations (segmental aneuploidy). Mutations or altered expression of CIN and mitotic genes are linked with the origin and evolution of aneuploidy, but the precise molecular mechanisms and the therapeutic and diagnostic challenges are still not well defined (24–30). A common hypothesis is that CIN and aneuploidy, inferred from gene expression profiles, predict clinical outcome in multiple human cancers including OSCCs (31), in agreement with early reports in which aneuploidy was evaluated by DNA flow cytometry (DNA-FCM; refs. 32, 33). This hypothesis does not exclude, however, that aneuploidy represents an early event in premalignant lesions (19–23, 34) including OPMDs (20–22, 35, 36). New prospective studies should address the relevance of aneuploidy to the field effect cancerization in ONAMFs and in OPMDs with or without dysplasia (3, 4, 11, 37, 38) taking into account that the rate of OPMD progression to OSCCs is only about 1.3% per year (39) and that, remarkably, OSCCs may develop either within the area of the OPMDs or in ONAMFs (6). DNA aneuploidy detected by image cytometry (40) was used to predict malignant transformation of OPMDs in 3 important but relatively small retrospective studies using formalin-fixed paraffin-embedded material (41–43). Dysplastic OPMDs progressing into OSCCs had a statistically significant higher frequency of DNA aneuploidy than the dysplastic nonprogressing OPMDs (41, 43). The third study, investigating both nondysplastic and dysplastic OPMDs, confirmed the prognostic value of DNA aneuploidy with OR of 7.1 and $P = 0.008$ (42). Though these studies were in good agreement with each other, the significance of DNA aneuploidy remained relatively uncertain, and clearly novel larger prospective studies are needed. In the present prospective study, which included ONAMFs and nondysplastic and dysplastic OPMDs, we evaluated the DNA index (DI) by high-resolution flow cytometry (hr-DNA-FCM; ref. 35) and investigated the association of the DI values with high-risk oral sites (tongue and floor of the mouth; FOM; ref. 15) and dysplasia in OPMDs as surrogate endpoints of risk of OSCC development.

Materials and Methods

Patients

Patients with OPMDs were recruited during the period 2009 to 2012 in the Oral Medicine and Oral Oncology Section of the University of Turin at the A.O.U.S. Luigi Gonzaga (Orbassano, Turin). Patient written consent was obtained in every case during an interview according to the Institutional Ethic Committees (A.S.O.S. Luigi Gonzaga Prot. N. 11780). Classical clinical data, including tobacco and alcohol habits, were recorded. Current tobacco cigarette smokers (71/164, 43%) were almost all characterized by heavy smoking (more than 10 cigarettes/day). Former smokers, who quitted smoking at least 6 months before the interview, entered a third category (39/164, 24%) beside the nonsmokers (54/164, 33%). Alcohol high exposure (abuse) was considered at the consumption of at least 2 or more than 2 alcoholic units (AUs) per day. Consumption of 1 AU (equivalent to about 10 grams of ethanol or 1 glass of wine or 0.25 L of beer or 1 measure of liquor) or less than 2 AUs was considered light to moderate exposure without clear risk. Patients, who did not provide adequate information, were not included in the statistical analyses. All patients were characterized by the presence of at least one OPMD. Patients with multiple OPMDs were included. The total number of consecutive patients, after excluding the patients with previous or present OSCCs or corresponding to degraded OPMD samples, which did not allow accurate DNA content histograms by hr-DNA-FCM, was 165. A subgroup of nonselected 132 patients included in this series gave written consent also for a curette sampling from ONAMFs. The total number of ONAMF samples was 135. In addition, 36 young individuals without oral lesions and tobacco cigarette smoking habit, who underwent surgery for the extraction of wisdom teeth, consented to be donors of "true" normal oral mucosa. According to a large prospective clinical study, which validated the tongue and FOM subsites as risk predictors for progression to oral cancer of mild/moderate dysplastic OPMDs (17), we considered dysplasia (irrespective of the degree) and these 2 subsites as surrogate endpoints of a high-risk group of patients. The low-risk group comprised patients with OPMDs without dysplasia and not located in these subsites.

Oral tissue sampling and histology

ONAMFs and OPMDs were characterized by their oral cavity subsites (tongue, FOM, buccal mucosa, gum, lip, hard- and soft palate). All the ONAMF samples were obtained within the same subsite of the reference OPMD with the use of a disposable curette as detailed elsewhere (44). In particular, care was taken to cause a slight bleeding from the ONAMFs to ensure that the basal layers of the epithelium had been collected. This sampling procedure was not as invasive as a punch biopsy and provided, in most cases, microbiopsies in a sufficient quantity to investigate the absence or presence of dysplasia (44) and conduct the hr-DNA-FCM analysis. All the OPMD samples, instead, were normally obtained by both punch biopsy and curette. When the OPMD size was comparable with the size of biopsy, only the punch biopsy was conducted. The samples were subdivided for formalin fixation, assessment of dysplasia by routine hematoxylin and eosin staining, and for immediate storage at $-20^{\circ}C$ for later measurements by hr-DNA-FCM. The diagnosis of OPMDs was based on internationally accepted criteria with the levels of diagnostic certainty C3–C4 (45).

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The histologic diagnosis for the assessment of dysplasia was carried out by a specially trained pathologist, according to the World Health Organization guidelines (46).

High-resolution DNA flow cytometry

Details of the methods are reported elsewhere (35). In brief, fresh/frozen tissue fragments were minced on Petri dishes using scalpels, collected in 2 mL detergent solution (0.1 mol/L citric acid, 0.5% Tween-20), and then submitted to mechanical disaggregation in a disposable 50 μm Medicon using a Medimachine (DAKO). Nuclei suspensions were obtained and filtered over a 50-μm nylon sieve (CellTrics, Partec GmbH). An absolute count of the nuclei in suspension, based on blue laser perpendicular and forward light scattering, was conducted by FCM (CyFlow ML, Partec GmbH) after 1 to 10 dilutions in water. The final volume was calculated to obtain the concentration of about 600,000 nuclei/mL. One volume (1/7 of the final volume) of detergent solution was first added followed by 10 minutes incubation. Finally, 6 volumes (6/7 of the final volume) of staining solution [0.4 mol/L Na_2HPO_4, 5 μmol/L 4′, 6-diamidino-2-phenylindole (DAPI) in water] were added. Samples were kept in dark for a minimum of 15 minutes incubation before the measurements by hr-DNA-FCM. 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 hr-DNA-FCM, quality controls, and DNA content histogram analysis were conducted according to consensus criteria (47). Only the samples with at least 2 separate G_0–G_1 peaks were considered DNA aneuploid. Gender-specific "true" oral normal mucosa from healthy donors and/or normal human lymphocytes were used as DNA diploid controls. The degree of DNA aneuploidy (DI ≠ 1), expressing the amount of abnormal DNA content relative to normal, was calculated as the ratio of mean channel number of the DNA aneuploid G_0–G_1 peak(s) to the mean channel number of the DNA diploid G_0–G_1 peak. DNA diploidy had DI = 1. The coefficient of variation (CV) values of the G_0–G_1 peaks for the DNA diploid "true" oral normal mucosa samples were used as a measure of accuracy (DNA resolution). A mean CV value of 1.9 ± 0.5% was obtained by Gaussian curve fitting (FloMax Software 3.0b4 2001, Partec GmbH). A mean value of 1.5 ± 0.6% was obtained by dividing the peak width at half maximum (in channel number) by the peak mean channel and the factor 2.35. The mean CV values obtained by the 2 methods when using normal human lymphocytes were, respectively, 1.2 ± 0.2% and 0.9 ± 0.2%.

Statistical analysis

Data collection and management were conducted using the Microsoft Office Excel package in association with the SPSS 16.0 software package (SPSS Inc.) for the statistical analyses. The total numbers of analyzed ONAMFs and OPMDs have been, respectively, 135 and 195 corresponding to 132 and 165 patients. Twenty-four patients had multiple OPMDs in different subsites: 20 had 2 OPMDs, 3 had 3 OPMDs, and 1 had 5 OPMDs. The patients with OPMDs in multiple subsites were represented by the worst OPMD characteristics of dysplasia and/or location in the 2 high-risk subsites (tongue and/or FOM) and/or DNA aneuploidy (DI≠ 1). OPMDs with more than one DNA aneuploid subline were assigned to a specific DI group with multiple DIs≠ 1. According to these criteria,

Table 1. Association of patient characteristics with patient low- and high-risk groups[a]

Patient characteristics	Number of patients	Low risk (n = 99)	High risk (n = 66)	OR (95% CI)	Two-tailed P
Age, y					
Young (<61.6)	83	53 (64%)	30 (36%)	1	
Old (>61.6)	82	46 (56%)	36 (44%)	1.4 (0.74–2.6)	0.34
Gender					
Female	85	48 (56.5%)	37 (43.5%)	1	
Male	80	51 (64%)	29 (37%)	0.74 (0.40–1.4)	0.42
Tobacco habit					
Nonsmokers	54	30 (63%)	24 (37%)	1	
Former smokers	39	25 (64%)	14 (36%)	0.70 (0.30–1.6)	0.52
Current smokers	71	44 (62%)	27 (38%)	0.77 (0.37–1.6)	0.58
Alcohol exposure					
No-light-moderate	87	50 (58%)	37 (42%)	1	
High[b]	47	29 (62%)	18 (38%)	0.84 (0.4–1.7)	0.71

[a]The high-risk group definition was based on the presence of dysplasia and/or high-risk subsites of tongue or FOM according to the prospective study of Zhang and colleagues (17) and the study of Castagnola and colleagues (15). The low-risk group was characterized by nondysplastic OPMDs located in the low risk sub-sites (other than tongue or FOM).
[b]Consumption of 2 or more than 2 AUs (see Materials and Methods).

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we obtained a group of 66 patients at high risk and a group of 99 patients at low risk with OPMDs without dysplasia or located in all the other subsites different from tongue and FOM. The association of gender, age, tobacco cigarette smoking, and alcohol abuse with the low- and high-risk patient groups was evaluated by 2×2 contingency tables with the 2-sided P values according to the Fisher exact test. To evaluate the association of the DI values versus the low- and high-risk patient groups, we conducted a multivariate logistic regression including patient gender, age, tobacco cigarette smoking habit, and alcohol abuse as confounding variables. OR values were reported together with the 95% confidence intervals (CI). $P < 0.05$ was considered as statistically significant.

Results

The present prospective study included 165 consecutive patients with at least one OPMD and without present or past history of OSCC. The patient characteristics are reported in Table 1. The recruited patients were 85 women and 80 men with, respectively, median ages of 65.7 and 59.7 years (age ranges of 27–93 and 26–86 years). Age was subdivided in 2 classes (young/old), respectively, below and above the median age (61.6 years). Tobacco cigarette smoking habit information was available for 164 patients (54 were nonsmokers, 33%; 39 were former smokers, 24%; 71 were current smokers, 43%). Alcohol exposure information was available for 134 patients. The association of the patient features represented by age, gender, tobacco cigarette smoking habit, and alcohol abuse with the low- and high-risk groups (see Materials and Methods) was first investigated using 2×2 contingency tables. None of these patient features was associated with the low- and high-risk groups (Table 1). In particular, we also explored the association between smoking (nonsmokers vs. former and current smokers) and dysplasia. No statistically significant association was observed (OR = 0.65; $P = 0.35$; data not shown).

High-resolution DNA-FCM was conducted on nuclei suspensions obtained from ONAMF and OPMD tissue samples to evaluate the nuclear DNA content and the corresponding DI values, as exemplified in Fig. 1. Figure 1A shows a DNA near-diploid aneuploid peak with DI = 1.02, which corresponds to a DNA change above the DNA diploid content (DI = 1.00) of 2%. Figure 1B shows 2 DNA aneuploid sublines with DIs of 1.18 and 1.22 (DNA change of 3%) within the same OPMD.

The percent incidences of DNA aneuploidy among "true" normal oral mucosa from healthy donors (n = 36), ONAMFs (n = 135), OPMDs without dysplasia (n = 171), and with dysplasia (n = 24), were respectively, 0%, 15%, 19%, and 38% (Fig. 2A). The incidences of multiple DNA aneuploid sublines in ONAMFs, nondysplastic OPMDs, and dysplastic OPMDs were, respectively, 0%, 1.8%, and 8.3% (data not shown).

The DI aneuploid values were subdivided in DNA near-diploid aneuploid (DI ≠ 1 and <1.4) and high aneuploid (DI ≥ 1.4). We found that the frequencies of the high-aneuploid (DI ≥ 1.4) sublines among the ONAMFs, nondysplastic, and dysplastic OPMDs were, respectively, 5%, 17%, and 45% (Fig. 2B).

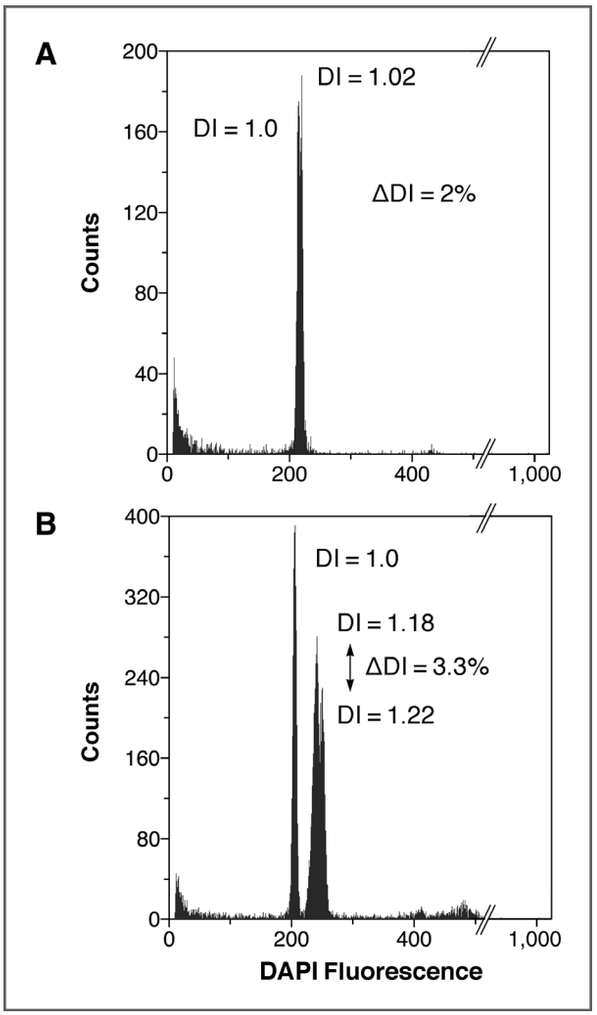

Figure 1. Examples of DNA near-diploid aneuploid sublines as obtained by hr-DNA-FCM (35, 36). A, in ONAMF. B, in OPMD.

Table 2 reports the results obtained by 2×2 contingency table and logistic regression analyses for assessing the association between the DI values obtained from ONAMFs in132 patients with OPMDs and the low- and high-risk patient groups (see Statistical analysis in Materials and Methods). The DI values were first subdivided in 2 groups only corresponding, respectively, to DNA diploid (DI = 1) and DNA aneuploid cases (DI ≠ 1). Seventy-two out of the 112 patients (64%) with DNA diploid ONAMFs occurred in the low-risk group, whereas 14 out of 20 patients (70%) with DNA aneuploid ONAMFs turned out to belong to the high-risk group (OR = 4.2; 95% CI, 1.5–12; $P = 0.006$). A logistic regression, conducted after including the same patients and taking gender, age, and tobacco cigarette smoking habit as covariates,

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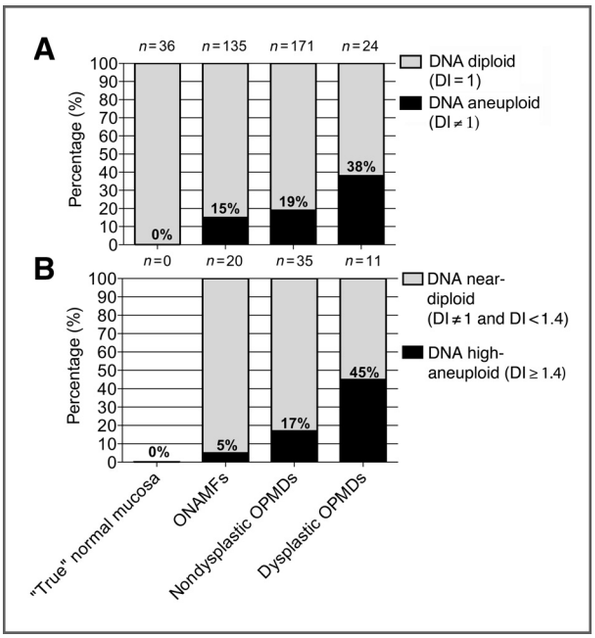

Figure 2. Frequencies of DI values among "true" normal oral mucosa, ONAMFs, and nondysplastic and dysplastic OPMDs.

improved the previous association results (OR = 5.3; 95% CI, 1.8–16; $P = 0.003$; Table 2). The inclusion of alcohol abuse for a reduced number of 107 patients with available data was also explored (see Materials and Methods). The results obtained (OR = 5.8; 95% CI, 1.7–20; $P = 0.006$; data not shown) were very close to the ones reported in Table 2, which did not include alcohol consumption.

Table 3 reports the association analyses of the patients with OPMDs subdivided in DI groups versus the low- and high-risk patient groups. The results obtained by 2 × 2 contingency table analysis among 165 patients with OPMD indicate that the patients with a single-DNA near-diploid aneuploid subline (with DI ≠ 1 and DI < 1.4) in their OPMDs had 3.6-fold increase in risk (95% CI, 1.5–8.6; $P = 0.004$) compared with the patients with the DNA diploid OPMDs. The OR value corresponding to the patients with high- or multiple DNA aneuploid OPMDs (DI ≥ 1.4 or multiple DIs ≠ 1) was characterized by OR = 8.5 (95% CI, 1.7–42; $P = 0.004$). One may notice that the patients characterized by OPMDs with high-DNA aneuploid or multiple DNA aneuploid sublines were associated with the "high-risk group" in 8 out of 10 cases (80%). The patients with DNA diploid and near-diploid aneuploid OPMDs were, instead, associated with the "high-risk" group in, respectively, 32% and 63% of the cases. A logistic regression was conducted including 164 patients with OPMD (one patient was lost due to absence of information for tobacco use) taking gender, age, and tobacco smoking habit as covariates. The results obtained improved slightly with respect to the previous analysis conducted by 2 × 2 contingency table providing, respectively, OR = 4.3 (1.7–11), $P = 0.002$ and OR = 9.2 (1.8–47), $P = 0.007$. We also explored by logistic regression analysis the changes introduced by including the alcohol abuse information, which was available for 134 patients. The results were very close to the previous ones (data not shown).

Table 4 reports the results obtained by 2 × 2 contingency table for the association between DI groups determined from ONAMFs, available for 132 patients, and OPMDs, available for 165 patients, with the patient low- and high-risk groups. The results obtained by logistic regression with age, gender, and tobacco habit as confounding variables were slightly better than those obtained by 2 × 2 contingency table analysis: OR = 4.3 (1.8–10) and OR = 18.4 (3.8–89) with, respectively, $P = 0.001$ and $P < 0.0005$ (Table 4). The same analysis conducted after including the alcohol abuse information led to results very close to the previous ones (data not shown).

To make the data set more homogenous, we also explored the effects of reducing the OPMD number of cases to 132 in correspondence with the same 132 patients with available ONAMFs. The OR values, obtained after applying the previous logistic regression model based on the same DI groups, were respectively, 5.5 and 55.3 (data not shown).

Discussion

The role of CIN and aneuploidy in the genesis and progression of oral premalignancy (25–29) and, in particular, in their link with the concept of "oral field effect

Table 2. Association of 2 DI groups (with DI = 1 and DI≠1) in ONAMFs for 132 patients with OPMDs with patient low- and high-risk groups[a]

	Risk groups				
DI groups	Low risk	High risk	ORs (by LR)[b]	95% CI (by LR)[b]	*P* (by LR)[b]
DI = 1	72 (64%)	40 (36%)	Reference group (OR = 1)		
DI ≠ 1	6 (30%)	14 (70%)	4.2 (5.3)	1.5–12 (1.8–16)	0.006 (0.003)

[a]The low- and high-risk group definitions are reported in Materials and Methoda and in the legend of Table 1.

[b]By LR OR, 95% CI, and 2-tailed Fisher *P* values reported in parentheses were estimated by logistic regression (LR) after adjustment for gender, age, and tobacco cigarette smoking habit as confounding variables. The other OR, 95% CI, and *P* values were obtained by 2 × 2 contingency table analysis taking the DNA diploid group (DI = 1) as reference group with OR = 1.

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Table 3. Association of 3 groups of DI values corresponding to 165 OPMDs with the low- and high-risk patient groups[a]

	Risk groups				
DI groups	Low risk	High risk	ORs (by LR)[b]	95%CI (by LR)[b]	*P* (by LR)[b]
DI = 1	87	41 (32%)	Reference group (OR = 1)		
DI ≠ 1 and DI < 1.4	10	17 (63%)	3.6 (4.3)	1.5–8.6 (1.7–11)	0.004 (0.002)
DI ≥ 1.4 or multiple DIs ≠ 1	2	8 (80%)	8.5 (9.2)	1.7–42 (1.8–47)	0.004 (0.007)

[a]The low- and high-risk group definitions are reported in Materials and Methods and in the legend of Table 1.

[b]By LR OR, 95% CI, and 2-tailed Fisher *P* values reported in parentheses were estimated by logistic regression (LR) after adjustment for gender, age, and tobacco cigarette smoking habit as confounding variables in 164 patients with OPMD (one patient was lost due to absence of information for tobacco use). The other OR, 95% CI, and *P* values were obtained by 2 × 2 contingency table analysis taking the DNA diploid group (DI = 1) as reference group with OR = 1.

cancerization," is still relatively uncertain. The field effect theory postulates that an initial oral patch, which is not visually recognizable but characterized by stem cells sharing genetic/genomic aberrations, is converted into an expanding field with numerous aberrations. This field might become then visible as leukoplakia or erythroplakia or OPMD (3, 37, 48–51). OPMDs, which may be nondysplastic and dysplastic, were characterized by a relatively low rate of transformation of about 1.3% per year (39). Moreover, OPMDs are not the only precursors of OSCCs (2–6, 52) because OSCCs may also develop from ONAMFs characterized by absence of dysplasia and with a transformation rate that still remains to be evaluated. The genomic characteristics of ONAMFs and OPMDs were recently investigated using array-comparative genomic hybridization and hr-DNA-FCM in a small number of cases (15, 16). Presently, 135 ONAMFs and 195 OPMDs (171 nondysplastic and 24 dysplastic) for a consecutive series of 165 patients with OMPD were investigated by hr-DNA-FCM.

The DNA aneuploid ONAMFs and DNA aneuploid nondysplastic OPMDs were characterized by DNA near-diploid aneuploid sublines (DI ≠ 1 and < 1.4) in, respectively, 95% and 83% of the cases. These data suggest that the detected DNA near-diploid aneuploid sublines represent early events of the natural history of oral cancer genesis and progression. We speculate that these DNA aberrant sublines, which had DNA changes below 20% with respect to DNA diploidy in the vast majority of the cases, were likely to be concomitant with genetic, epigenetic, and genomic aberrations, as reported in the literature (2–4, 6, 11, 13, 50, 52), and that some of these aberrations contributed to induce CIN and aneuploidy. Clearly, no cause–effect relationships can be drawn from the present data; specific experiments, including *in vitro* and mouse models, are necessary toward these purposes. We have also observed that the DNA aneuploid OPMDs with dysplasia were often (45%) characterized by high-DNA aneuploidy (DI ≥ 1.4) and/or by multiple DNA aneuploid sublines (22%).

We think that these characteristics, including the subsites of the tongue or FOM among the ONAMFs and OPMDs, may represent predictors of high risk of cancer. To validate this hypothesis, however, one clearly needs the knowledge of the clinical endpoint of cancer development. These data, which are difficult to obtain, due to the low rate of transformation of both OPMDs and ONAMFs, were not available at this time. We were

Table 4. Association between 3 groups of DI values determined from ONAMFs, available for 132 patients, and OPMDs, available for 165 patients, with the patient low- and high-risk groups[a]

	Risk groups				
DI groups	Low risk	High risk	ORs (by LR)[b]	95%CI (by LR)[b]	*P* (by LR)[b]
DI = 1	84	34 (28%)	Reference group (OR = 1)		
DI ≠ 1 and DI < 1.4	13	19 (59%)	3.6 (4.3)	1.6–8.1 (1.8–10)	0.003 (0.001)
DI ≥ 1.4 or multiple DIs ≠ 1	2	13 (87%)	16.1 (18.4)	3.4–75 (3.8–89)	0.00002 (<0.0005)

[a]The low- and high-risk group definitions are reported in Materials and Methods and in the legend of Table 1.

[b]By LR, OR, 95% CI, and 2-tailed Fisher *P* values reported in parentheses were estimated by logistic regression (LR) after adjustment for gender, age, and tobacco cigarette smoking habit as confounding variables. The other OR, 95% CI, and *P* values were obtained by 2 × 2 contingency table analysis taking the DNA diploid group (DI = 1) as reference group with OR = 1.

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obliged, therefore, to investigate the prognostic relevance of the DI values only by testing their association with 2 patient groups at different risk classified according to presence/absence of dysplasia and/or subsite position (see Materials and Methods). In practice, we used 2 intermediate surrogate endpoints of risk of cancer, the first one being the presence of dysplasia in the OPMDs. Dysplasia in OPMDs, though limited for a set of problems that were amply discussed in the literature (53–56), is still the golden reference standard for the patient treatment. The second surrogate endpoint was based on the subsite of the OPMDs considering that the tongue and FOM subsites were qualified predictors of risk of cancer development in a recent study involving a prospective cohort of 296 patients with OPMDs with mild/moderate dysplasia (17, 57, 58). Distinctive features of chromosomal aberrations and increased incidence of DNA aneuploidy for the tongue OPMDs with respect to the OPMDs in all the other oral subsites were also reported in a recent study from our group, which suggested that these patients should receive a distinctive special attention (15).

In the present series of 165 patients, the numbers of OPMDs and ONAMFs were reduced, respectively, to 165 and 132 (see Statistical analysis in Materials and Methods). A logistic regression analysis, including gender, age, and tobacco cigarette smoking habit taken as covariates, showed that the patients with OPMD DNA near-diploid aneuploid (DI $\neq 1$ and <1.4) and high (DI $\geq$ 1.4) or multiple DNA aneuploid sublines versus the DNA diploid cases (DI $= 1$; OR $= 1$) had, respectively, a 4.3- and 9.2-fold increase (corresponding to $P = 0.002$ and $P = 0.007$) to belong to the high-risk groups (see Statistical analysis for the definition of low- and high-risk patient groups and the results reported in Table 3). In particular, when we included the DIs from both ONAMFs and OPMDs, the patients with DNA near-diploid aneuploid (DI $\neq 1$ and <1.4) and high- (DI ≥ 1.4) or multiple DNA aneuploid sublines were characterized by 4.3- and 18.4-fold increases to belong to the high-risk group (95% CIs, 1.8–10 and $P = 0.001$; 95% CIs, 3.8–89 and $P < 0.0005$; Table 4). Moreover, when we restricted the same analysis to a more homogeneous data set of 132 patients with corresponding 132 OPMDs and ONAMFs, the ORs improved, respectively, to 5.5 and 55.

We then explored by logistic regression analyses the possible changes introduced by alcohol abuse in the results obtained after adjustment with age, gender, and tobacco habit taken as confounding variables. It is of notice that the alcohol consumption responders were sometimes contradictory and not considered fully reliable. The analyses were done, therefore, with a reduced number of patients: 134 among 165 with OPMD samples and 107 out of 132 with ONAMF samples. We found that the inclusion of alcohol abuse information together with age, gender, and tobacco smoking habit introduced only minimal changes. In all cases, the P values were above 0.2 for all these confounding variables. Age, gender, tobacco smoking habit, and alcohol abuse, therefore, had no significant impact in the analysis of our data set, and the DI values were clearly shown as independent risk factors.

The present data indicate that the DI values detected by hr-DNA-FCM in ONAMFs and OPMDs for patients with OPMDs may represent biomarkers potentially useful to stratify oral premalignancy patients into new low- and high-risk groups for the development of OSCCs. Therefore, the presence of dysplasia in OPMDs, their position in the high-risk oral subsites (tongue and FOM), the detection of DNA near-diploid aneuploid and high- or multiple DNA aneuploid sublines in both ONAMFs and OPMDs may potentially help to improve the diagnosis and treatment of patients with oral premalignancy.

The present study also showed that our optimized DNA-FCM methodology seemed appropriate to detect, at high resolution and in the clinical routine setting, the presence and multiplicity of DNA near-diploid aneuploid sublines in normal-appearing mucosa and nondysplastic OPMDs (35, 36). These DNA aneuploid sublines could, in fact, be properly measured also in critical conditions such as slight increase or decrease of DNA content between 2% and 5% and small percentages of nuclei (between 2% and 5%) in the DNA aneuploid sublines (35).

Overall, we think that the presence of DNA aneuploid sublines in both the OPMDs and ONAMFs may be potentially useful for a closer follow-up and treatment of the patients at high risk of OPMD cancerization and risk of a new carcinoma in a field of preneoplastic cells. Clearly, validation of the clinical value of this approach, while conducting large-scale prospective studies with accurate follow-up data and clinical endpoints, remains to be done.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W. Giaretti
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Monteghirfo, M. Pentenero, S. Gandolfo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Giaretti, S. Monteghirfo, D. Malacarne, P. Castagnola
Writing, review, and/or revision of the manuscript: W. Giaretti, S. Monteghirfo, P. Castagnola
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Monteghirfo, D. Malacarne
Study supervision: W. Giaretti

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