Predicting Progression of Low-Grade Oral Dysplasia Using Brushing-Based DNA Ploidy and Chromatin Organization Analysis

Madhurima Datta[1,2,3], Denise M. Laronde[1,2], Miriam P. Rosin[2,4], Lewei Zhang[1,2,5], Bertrand Chan[1,2], and Martial Guillaud[3,6]

ABSTRACT

Most oral cancers arise from oral potentially malignant lesions, which show varying grades of dysplasia. Risk of progression increases with increasing grade of dysplasia; however, risk prediction among oral low-grade dysplasia (LGD), that is, mild and moderate dysplasia can be challenging as only 5%–15% transform. Moreover, grading of dysplasia is subjective and varies with the area of the lesion being biopsied. To date, no biomarkers or tools are used clinically to triage oral LGDs. This study uses a combination of DNA ploidy and chromatin organization (CO) scores from cells obtained from lesion brushings to identify oral LGDs at high-risk of progression. A total of 130 lesion brushings from patients with oral LGDs were selected of which 16 (12.3%) lesions progressed to severe dysplasia or cancer. DNA ploidy and CO scores were analyzed from nuclear features measured by our in-house DNA image cytometry (DNA-ICM) system and used to classify brushings into low-risk and high-risk. A total of 57 samples were classified as high-risk of which 13 were progressors. High-risk DNA brushing was significant for progression ($P = 0.001$) and grade of dysplasia ($P = 0.004$). Multivariate analysis showed high-risk DNA brushing showed 5.1- to 8-fold increased risk of progression, a stronger predictor than dysplasia grading and lesion clinical features. DNA-ICM can serve as a non-invasive, high-throughput tool to identify high-risk lesions several years before transformation. This will help clinicians focus on such lesions whereas low-risk lesions may be spared from unnecessary biopsies.

Prevention Relevance: DNA ploidy and chromatin organization of cells collected from oral potentially malignant lesions (OPMLs) can identify lesions at high-risk of progression several years prior. This non-invasive test would enable clinicians to triage high-risk (OPMLs) for closer follow-up while low-risk lesions can undergo less frequent biopsies reducing burden on healthcare resources.

Introduction

Oral squamous cell carcinoma (SCC) is believed to arise through sequential stages of potentially malignant lesions (OPML), that is, hyperplasia, mild, moderate, severe dysplasia, and carcinoma *in situ* (1). The presence and grade of dysplasia are considered the gold standard to predict the risk of malignant transformation; risk increases with higher grades of dysplasia (2). The majority of high-grade dysplasia progress to cancer without treatment, whereas the majority of low-grade (mild and moderate) dysplasia (LGD) remain stable (3). As a result, treatment decisions are guided by the gold standard of histopathology with high-grade dysplasia requiring treatment. However, histopathology has several limitations. One, a clinician must biopsy the worst part of the lesion to determine the highest degree of dysplasia. For example, if a severely dysplastic lesion had a biopsy from an area of the lesion with only mild dysplasia, then the degree of dysplasia would not represent the cancer risk of the lesion (4). Two, grading of dysplasia is subjective, and studies have reported high rates of inter and intra-observer variability (5). Three, the degree of dysplasia does not reliably predict the risk of malignancy: Oral cancer can develop without dysplasia whereas lesions with severe dysplasia may remain stable over time (6). Risk prediction of LGDs, the majority of OPMLs diagnosed, is challenging, because only 5%–15% undergo malignant transformation (7).

There is no consensus as to the treatment of LGDs (8). In our longitudinal study, we found that if LGDs were treated conservatively, without a wide margin, the treatment did not lower the risk of malignancy (9). Consequently, in British Columbia, LGD is followed in specialty clinics, when possible, whereas severe

[1]Department of Oral Biological and Medical Sciences, Faculty of Dentistry, The University of British Columbia, Vancouver, British Columbia, Canada. [2]BC Oral Cancer Prevention Program, Cancer Control Research, Vancouver, British Columbia, Canada. [3]Imaging Unit, Integrative Oncology, BC Cancer, Vancouver, British Columbia, Canada. [4]Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, British Columbia, Canada. [5]Department of Laboratory Medicine and Pathology, BC Oral Biopsy Service, Vancouver General Hospital, Vancouver, British Columbia, Canada. [6]Department of Statistics, The University of British Columbia, Vancouver, British Columbia, Canada.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Corresponding Author: Martial Guillaud, Imaging Unit, Room 6–108, 675 West 10th Avenue, Vancouver, BC, V5Z 1L3, Canada. Phone: 604-6765-8086; Fax: 604-675-8099; E-mail: mguillau@bccrc.ca

Cancer Prev Res 2021;14:1111–8

doi: 10.1158/1940-6207.CAPR-21-0134

©2021 American Association for Cancer Research

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

dysplasia, carcinoma *in situ*, and SCC are treated with curative intent. Oral LGDs consist of lesions that are at both high- and low-risk of progression; hence, they have a very varied natural history of disease with time to progression ranging widely from 7 to 177 months (10). On one hand, LGDs with high cancer risk may not have an appropriate intervention until they have progressed to cancer. On the other hand, LGDs with low cancer risk could be subjected to repeated biopsies that may lead to undue morbidity at the lesion site (11). Biomarkers are needed to triage LGDs according to their risk of progression.

Currently, clinicians depend on clinical risk indicators like size, appearance, and site to determine the cancer risk of OPMLs as no biomarkers or diagnostic tools are available clinically to guide triage or treatment strategies (12). Studies have been ongoing to identify biomarkers that can help identify lesions at a high-risk of progression (13).

DNA aneuploidy has shown to be a marker of various malignancies, including oral cancers (14). A normal cell has twice the basic set of 23 chromosomes and is referred to as diploid whereas cells that do not possess an integer multiple of the basic set of chromosomes are referred to as aneuploid (15). Errors in cell division and DNA replication (chromosome segregation, centrosome amplification) often give rise to abnormal DNA content that fuels carcinogenesis (16). Most aneuploidies are fatal to the cell; however, a few viable aneuploid cells give rise to stable aneuploid cell lineages (17). Identification of these aneuploid clones early in the process of carcinogenesis could identify OMPLs at high-risk of progression.

DNA ploidy can be measured using a variety of assays. In this study, DNA image cytometry (DNA-ICM) is used to assess DNA ploidy as it is capable of detecting small percentages of aneuploid cells (18). DNA-ICM can be done using brushing or biopsy tissue samples. There is considerable clinical interest to develop a non-invasive tool for cancer diagnosis and prognosis. Brushings collect cells in a non-invasive manner and ensure representation of the entire lesion area, however brushings may fail to collect abnormal cells from deeper layers of the oral epithelium (19). DNA-ICM uses a DNA content histogram method to determine the DNA content of each nucleus in context to the cell cycle (17). DNA content of each nucleus is measured by a normalized scale referred to as DNA Index (DI). Samples typically show a dominant peak of normal diploid cells (DI = 1) and a smaller peak of tetraploid cells (D1 = 2) stalled at the G_2–M phase of cell division. If cell proliferation is high, cycling cells are seen between the diploid and tetraploid peak whereas cells showing DI > 2.3 are considered aneuploid. Aneuploid cells are a rare event and make up less than 1% of the total cell population. Studies have shown that tetraploidy is a precursor of aneuploidy (20). We believe that the percentage of non-diploid cells (cycling and tetraploid) in the absence of aneuploidy can also provide valuable information in identifying lesions at high-risk of progression.

In addition to changes in DNA amount, alterations in nuclear size, shape, and number, nucleoli shape, and chromatin distribution, provide additional information about the malignant nature of the cells. In cancer, nuclei become irregular and folded with increasing coarse heterochromatin aggregates (21). These changes in DNA organization have also been observed in diploid cells adjacent to cancerous fields as a result of genetic (activation of oncogenes) or epigenetic alterations (covalent modification of histones, methylation of DNA or chromatin modeling) that drive progression (22). Studies from our group have shown that DNA organization changes can serve as a prognostic marker in prostate, head and neck, and lung cancers (23–25). Using diploid cells to predict progression risk will be useful in absence of aneuploid cells in brushings due to sampling errors. In this study, we classify OPMLs as high-risk, or low-risk based on a combination of DNA ploidy and chromatin organization (CO) scores.

Oral cancer is a heterogeneous disease that arises as a result of accumulation of various genetic and epigenetic changes. DNA-ICM provides a unique opportunity to observe visual phenotypic changes in cells during oral carcinogenesis that could expand our understanding of the biological process. To date, all reported longitudinal studies have investigated the association of DNA aneuploidy with progression using oral biopsy tissue (26–32). Our study is the first to use lesion brushings to study DNA amount and CO changes to identify OPMLs at high-risk of progression.

Materials and Methods

Patient population

Patients were enrolled in an ongoing Oral Cancer Prediction Longitudinal (OCPL) study between 1997 and 2014. Oral LGD patients were referred by community clinicians for follow-up in our oral dysplasia clinics where they were invited to participate in the OCPL study. Study protocol and ethical approval were obtained from the University of British Columbia and BC Cancer Agency Research Ethics Board, and participants were recruited by written informed consent. For this retrospective study, inclusion criteria included patients with brushing samples concurrent or 12 months before biopsy showing mild or moderate dysplasia, or hyperplasia with a previous history of dysplasia. Majority of the brushings were obtained before the index biopsy; however, some brushings were obtained at subsequent 6-month follow up visits and matched with biopsy results 6–12 months following the brushing date. For large OPMLs, multiple biopsies were obtained to represent the true risk of the lesion and the worst biopsy diagnosis was considered in the study. Biopsy site selection was decided by the oral medicine specialist and adjunct tools such as Toluidine Blue staining were used to determine the high-risk sites. Depending on the site, majority of the samples were 4–5 mm in diameter and 1.5–2 mm in depth and obtained using a punch. Histopathological grading was done at the Oral Biopsy Service according to the WHO 2005 classification and each report was signed off by two pathologists and verified by our study oral pathologist (L. Z.) to reduce interobserver variability (6).

Patients with a previous history of oral cancer were excluded. Clinical, demographic, and risk-habit information of the included participants were obtained from study records. Outcome was defined as progression to severe dysplasia or higher at the same anatomical site as the primary biopsy.

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

Included participants were grouped into progressors and non-progressors based on outcome.

Sample preparation and imaging

Oral lesion brushings were collected and processed as described in our previous study (33). The brushings were cytospun onto slides and stained with Feulgen Thionin, a DNA stoichiometric stain. DNA content of each cell is measured by the integrative optical density, which is measured by DI. The slides were scanned using our semiautomatic *in-house* Getafics scanner (33). The imaging system has undergone quality assurance and follows the recommendations of the European Society of Analytical Cellular Pathology (34, 35). For this study, a cell was defined as diploid when DI ranged from 0.9 to 1.2, cycling cell from 1.25 to 1.75 and tetraploid cells from 1.80 to 2.25; aneuploid cells had a DI > 2.3. In addition to showing number of cells in each group, a gallery of individual cells is also available for visual inspection. The DNA-ICM results were manually inspected by the graduate student (M. D.) and cytotechnicians (AC or JK) in case of discrepancies, to ensure correct normalization and that debris or overlapping cells were being classified as junk.

DNA organization was determined by calculating the CO scores of diploid cells. Diploid cells from progressing and non-progressing lesions were grouped together and over 89 nuclear features were used to train a Random Forest (RF) classifier to discriminate between them (ref. 36; Supplementary Table S1). The statistical analyses for generation of the chromatin re-organization score was done using R statistical software (version 3.5.2).

Statistical analysis

Data analyses were done using the SPSS Version software 27 (SPSS, Inc.). Clinical, demographic, and risk habit information were compared between the progressing and non-progressing groups using the χ^2 test or Fischer's exact for categorical variables and t test or Mann–Whitney U test for continuous variables. The threshold for significance was set at $P < 0.05$ and all tests were two tailed. Sensitivity and specificity analyses were done to determine accuracy of aneuploidy in progression prediction in comparison with the gold standard histopathology. Time to outcome was calculated from date of index biopsy diagnosis to progression or last follow-up date if no progression occurred. Time-to-progression curves for the risk groups were plotted using Kaplan–Meier analysis and log rank test. Hazard ratios (HR) and the corresponding 95% confidence intervals (CI) were determined using the univariate followed by multivariate Cox proportional analysis.

Results

Patient and sample data

From the 149 participants identified from the study records, 19 were excluded due to lack of adequate brushing sample for DNA-ICM analysis. Of the 130 participants, 45 had mild dysplasia (D1), 45 moderate dysplasia (D2), and 40 hyperplasia (H) or lichenoid lesions (LM/LP) with a history of dysplasia at the same site at the time of brushing. Follow-up was ascertained using the BC OCPL database and central biopsy service records. Thirty-seven patients were lost to follow-up at our dysplasia clinic before the indicated 5-year follow up period. The centralized biopsy service records were inspected to ensure included participants did not undergo progression subsequent to loss of follow-up. Sixteen (12.3%) participants progressed, 7 (43.8%) to severe dysplasia (D3), 5 (31.3%) to verrucous carcinoma (VC), and 4 (25%) to SCC.

Table 1. Comparison of progressing and non-progressing patients according to demographic, risk habit, and clinicopathological information.

	All patients	Progressing (%)	Non-progressing (%)	*P*
Total	130	16	114	
Age at diagnosis				
Mean (years ± SD)	61.7 ± 10.9	60 ± 11.7	61.9 ± 10.9	0.819
Gender				
Male	63	9 (14.3)	54 (85.7)	0.506
Female	67	7 (10.4)	60 (89.6)	
Ethnicity				
White	113	14 (12.4)	99 (87.6)	0.942
Other	17	2 (11.8)	15 (88.2)	
Ever smoker[a]				
No	41	6 (14.6)	35 (85.4)	0.600
Yes	88	10 (11.4)	78 (88.6)	
Ever consumed alcohol[b]				
No	52	6 (11.5)	46 (88.5)	0.979
Yes	77	9 (11.7)	68 (88.3)	
Risk site[c]				
Low risk	42	3 (7.1)	39 (92.9)	0.216
High risk	88	13 (14.8)	75 (85.2)	
Lesion size >200				
No	67	3 (4.4)	64 (95.6)	**0.005**
Yes	63	13 (20.6)	50 (79.4)	
Lesion appearance[d]				
Homogenous	66	7 (10.6)	59 (89.4)	0.291
Non-homogenous	52	9 (17.3)	43 (82.7)	
Ever TB positive[e]				
No	70	3 (4.3)	67 (95.7)	**0.003**
Yes	60	13 (21.7)	47 (78.3)	
FV Status[f]				
FVL	76	12 (15.8)	64 (84.2)	0.482
FVR	32	3 (9.4)	29 (90.6)	
Histology of index biopsy[g]				
Ever D1/LD1/H	70	3 (4.3)	67 (95.7)	**0.003**
Ever D2/LD2	60	13 (21.7)	47 (78.3)	
Months follow up/progression				
Mean (months ± SD)	91.3 ± 47	82.8 ± 50.2	92.4 ± 46.7	0.917

[a]Never smoker 0–100 cigarettes in a lifetime, ever smoker >100 cigarettes in a lifetime, data missing for 1 person.
[b]1 people had missing alcohol data.
[c]High-risk site, tongue and floor of the mouth; low-risk site, rest of the sites.
[d]12 lesions had missing appearance information.
[e]TB-positive anytime during follow-up of lesion.
[f]FVL, fluorescence visualization loss; FVR, fluorescence visualization retention; 17 lesions showed equivocal and 5 masking.
[g]D1, mild dysplasia; D2, moderate dysplasia; LD1, mild dysplasia with lichenoid features; LD2, moderate dysplasia with lichenoid features; H, hyperplasia.

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

Two of the 16 patients who underwent progression were identified from the centralized biopsy service records several years subsequent to 5-year follow-up. Breakdown between histological diagnosis and outcome of the cases in Supplementary Table S2.

Comparison of demographics, risk habit information, and lesion clinical features is also found in **Table 1**. There were no significant differences in age of diagnosis, sex, ethnicity, smoking and alcohol habit between the two groups. Time to progression ranged from 11 to 217 months (mean = 82.8, SD = 50.2) whereas length of follow-up at our dysplasia clinic among non-progressors ranged from 7 to 248 months (mean = 92.4, SD = 46.7). No significant differences were noted in the months of follow-up and progression between non-progressors and progressors. Higher degree of dysplasia (D2) was highly significant ($P = 0.003$) for progression. Lesion area greater than 200 mm^2 was also significant for progression ($P = 0.005$). Toluidine blue findings were noted at each follow-up visit and were coded as ever TB positive during the entire follow-up period. Ever TB positivity was significantly associated with progression ($P = 0.003$).

DNA ploidy score

Distribution of number of aneuploid cells (DI ≥ 2.3) and percentage of cycling (1.25 ≤ DI ≤ 1.75), and tetraploid cells (1.8 ≤ DI ≤ 2.25) were noted among progressors and non-progressors. A significantly higher percentage of tetraploid ($P = 0.005$) and cycling cells ($P = 0.044$), and number of aneuploid cells ($P = 0.044$) were noted among progressors versus non-progressors. As shown in previous studies, a two-step algorithm was used to determine the threshold that best differentiates progressors from non-progressors (37). Step 1 of the algorithm defined samples as high-risk when it contained one or more aneuploid cell (DI ≥ 2.3). Step 2 tested percentage of cycling, tetraploid, and cycling and tetraploid together using ROC curves to determine which combinations yielded the highest AUC. Percentage of cycling and tetraploid cells (cell proliferation) yielded an AUC of 0.72. A cutoff value of ≥1.9% cycling and tetraploid cells in addition to presence of 1 or more aneuploid cells showed an optimal sensitivity of 56% and specificity of 83%. Using the two-step algorithm, 28 samples showed high DNA ploidy scores, of which 9 were progressors. Samples were denoted as 1 or 0 depending on high or low DNA ploidy scores. High DNA

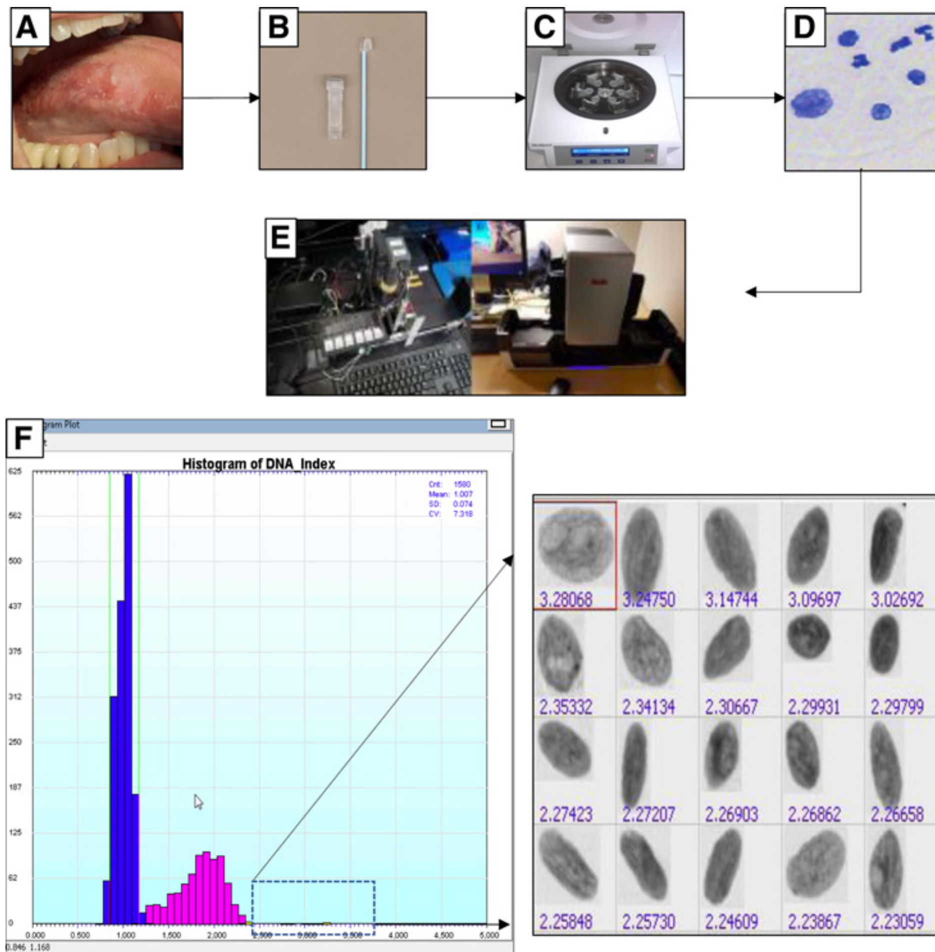

Figure 1.
A, A 44-year-old female patient presented with a mixed red and white lesion on the lateral tongue. Histological diagnosis showed moderate dysplasia. Lesion progressed to SCC 3 years later. **B–E,** DNA ploidy analysis was done on a brushing collected from the lesion. **F,** DNA histogram shows number of diploid cells (0.9 ≤ DI ≤ 1.2) in blue, cycling, and tetraploid cells (1.25 ≤ DI ≤ 2.25) in pink and aneuploid cells in yellow (DI ≥ 2.3). Image inset shows cell gallery of aneuploid cells with their respective DNA index value.

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

ploidy scores were found to be associated with lesion progression ($P = 0.002$). **Figure 1** shows a moderate dysplasia lesion with a large number of aneuploid cells, over 26 cells almost 3 years before progression.

Cell CO scores

A total of 127,450 diploid nuclei from non-progressors and 18,200 diploid nuclei from progressors were used for analysis. To balance the sets, 18,417 nuclei from non-progressors were randomly chosen. The nuclei were then randomly divided into a test and training set in a 50:50 ratio. Nuclear features of a total of 18,309 nuclei consisting of equal number of nuclei from progressing and non-progressing lesions were used to train the RF classifier (36). Once the parameters were tuned, the model was applied on the test set consisting of 18,308 nuclei. A classifying accuracy of 58% was obtained, which is similar to previous studies (38). Top classifying features were determined from the RF results. Fractal_dimen, a chromatin texture feature, was among the top 3 classifying features. Fractal_dimen is a measure of change in the pixel optical density integrated over the object at multiple resolutions, which is a quantitative method to approximate the ratio of euchromatin versus heterochromatin and its spatial distribution in the nucleus. CO score of each cell in the test set corresponds to the voting scores of the RF ranging from 0 (non-progressor-like) to 1 (progressor-like). CO score is a measure of how "progressor-like" or "non-progressor-like" each cell is within a sample. The mean cell CO scores for non-progressors and progressors were 0.47 ± 0.11 and 0.52 ± 0.11, respectively ($P < 0.001$).

Sample CO score

To obtain a sample score, a threshold score was determined at one, two, and three standard deviations from the mean CO score of progressors, that is, 0.6, 0.7, and 0.8. Percentage of cells in a sample with CO score at 0.6, 0.7, and 0.8 were calculated. This threshold was manually examined for optimal accuracy in predicting progression. Optimal sample CO score cutoff was determined to be 0.8 and lesions harboring cells above this threshold were considered to have a high CO score. Samples were denoted as 0 or 1 depending on their low or high CO scores.

DNA brushing classification

To increase the sensitivity of detecting high-risk lesions, 91 samples showing low DNA ploidy scores were evaluated for CO scores. A sample was classified as high-risk when DNA ploidy or CO score was 1 and classified as low-risk when both DNA ploidy and CO scores were 0. A total of 57 samples were classified as high-risk DNA brushing of which 13 were progressors. High-risk DNA brushings were associated to progression ($P = 0.001$) and higher grade of dysplasia ($P = 0.004$; **Table 2**). The sensitivity of detecting a lesion at high-risk of progression was 81% whereas specificity was 61%. High-risk DNA brushing had a high negative predictive value of 96% and a positive predictive value of 23%. Comparison of high-risk versus low-risk DNA brushing did not show association with demographic, risk habit or clinical feature information (Supplementary Table S3).

Table 2. Association of DNA brushing scores with histological diagnosis and progression.

	Total	Low-risk score (%)	High-risk score (%)	*P*
	130	73	57	
Histology				
D1/LD1/H[a]	70	48 (68.6)	22 (31.4)	**0.002**
D2/LD2[a]	60	25 (41.7)	35 (58.3)	
Outcome				
Progression	16	3 (18.8)	13 (81.3)	**0.001**
Non-progression	114	70 (61.4)	44 (38.6)	

Note: Row percentages are reported.
[a]D1, mild dysplasia; D2, moderate dysplasia; LD1, mild dysplasia with lichenoid features; LD2, moderate dysplasia with lichenoid features; H, hyperplasia.

Cox regression analysis and survival curves

High-risk patients had a shorter mean progression-free period of 177 months as compared with low-risk patients showing 216 months (log rank: $x^2 = 10.6$, df $= 1$, $P = 0.001$; **Fig. 2**). Univariate cox-regression analysis found lesions with high-risk DNA brushing had a 6.2-fold increased risk of progression than low-risk (95% CI, 1.8–21.9; $P = 0.004$; **Table 3**). Lesions with moderate dysplasia, ever stained TB positive and with area greater than 200 mm^2 also showed significant increased risk of progression. Multivariate analysis was done using combinations of these four variables (**Table 4**). In all three models the high-risk DNA brushing was the strongest predictor of progression with HRs ranging from 5.1 to 8.

Discussion

Previous studies from our laboratory showed that high-grade dysplasia and oral cancer show DNA amount abnormalities and DNA-ICM can differentiate them from normal and benign lesion with high sensitivity and specificity (37). On the basis of these results and biological relevance, we developed this algorithm using the combination of DNA ploidy and CO scores to identify oral LGDs at high-risk of progression. Our classification was tested using various combinations of DNA ploidy and CO score thresholds, but the performance of the system only showed marginal differences. Though our algorithm is based on a small sample size, it provides proof of concept that DNA brushing classification shows significant improvement in predicting risk of progression over dysplasia grading or clinical features alone.

The majority of previous studies evaluated DNA ploidy using biopsy tissue and combined it with dysplasia grading to determine OPMLs at risk of progression (26–32). They reported a sensitivity ranging from 33% to 62.5% and specificity ranging from 41% to 89%. It should be noted that all studies included severe dysplasia and reported higher predictive values

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

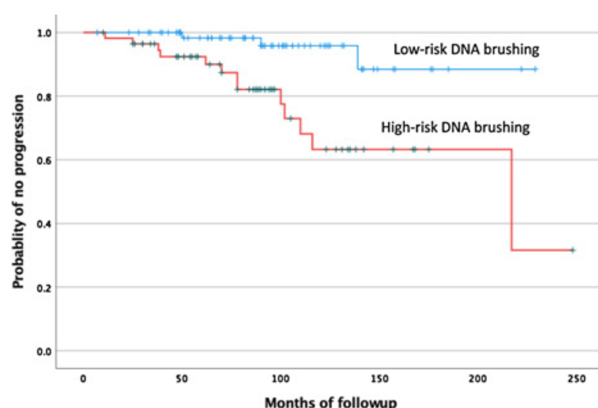

Figure 2.
Kaplan–Meier plot of time to progression comparing patients with high-risk DNA brushing with patients with low-risk DNA brushings. High-risk patients showed shorter progression-free mean period of 177 months as compared with 216 months for low-risk patients (log rank: $x^2 = 10.6$; df = 1; $P < 0.001$). Hazard ration is 6.1 (95% CI, 1.77–21.85).

Table 3. Univariate Cox regression analysis to predict lesion progression.

		Univariate[a]	
	N	**HR (95% CI)**	***P***
Total	130		
Age at diagnosis			
<61 years	66	1	0.211
≥61 years	62	0.52 (0.18–1.44)	
Gender			
Female	67	1	0.399
Male	61	0.65 (0.24–1.76)	
Ethnicity			
Other	16	1	0.970
White	112	0.97 (0.21–4.33)	
Ever Smoker			
No	40	1	0.672
Yes	87	1.2 (0.44–3.5)	
Ever consumed alcohol			
No	50	1	0.392
Yes	77	0.62 (0.21–1.8)	
Risk Site			
Low risk	40	1	0.339
High risk	88	1.84 (0.52–6.48)	
Lesion size >200			
No	67	1	**0.023**
Yes	61	4.31 (1.22–15.19)	
Lesion appearance			
Homogenous	66	1	0.242
Non-homogenous	50	0.55 (0.20–1.49)	
Ever TB positive			
No	69	1	**0.007**
Yes	59	5.68 (1.6–20.11)	
DNA brushing classification			
Low risk	72	1	**0.004**
High-risk	56	6.21 (1.77–21.85)	
Histology of index biopsy[b]			
Ever D1/LD1/H	68	1	**0.013**
Ever D2/LD2	60	4.96 (1.39–17.6)	

[a]2 non-progressors censored cases before the earliest event in a stratum.
[b]D1, mild dysplasia; D2, moderate dysplasia; LD1, mild dysplasia with lichenoid features; LD2, moderate dysplasia with lichenoid features; H, hyperplasia.

when DNA aneuploidy was combined with dysplasia grading. The higher progression rates of severe dysplasia may be responsible for the higher predictive values. A meta-analysis, which included five longitudinal studies, concluded that aneuploidy in oral biopsy tissue was associated with a 3.12-fold increased risk of progression to cancer (39). A previous study from our group found that high nuclear phenotype scores (a combination of five nuclear morphometric features that best discriminate cancer from normal cells) obtained from tissue samples were associated with OPML progression and high-risk molecular patterns (24). To our knowledge, this study is the first longitudinal study to use oral lesion brushings in place of biopsy tissue to predict progression. In our study, high-risk DNA brushing scores was associated with a 5.1- to 8-fold increased risk of progression. We believe that this algorithm of detecting single cell aneuploidies, increased proliferation and changes in CO in diploid cells collected using brushings is potentially more sensitive in detecting early changes in OPML that undergo progression. Even though it is believed that brushings are unable to collect abnormal cells from deeper layers of oral epithelium in LGDs, our findings show at least comparable results to previous studies using biopsy tissue.

Currently, in our dysplasia clinic, all oral LGD patients are followed at a 6-month interval with a few patients on a shorter recall depending on dysplasia grading and clinical risk factors as assessed by the oral medicine specialist. This can be challenging in community settings where high rates of intra-observer variability are seen. Also, it is often difficult for clinicians to determine the worst area of a lesion to biopsy. A recent large population-based retrospective cohort study highlighted the shortcomings associated with triaging of OPMLs based on histopathological diagnosis alone and the lack of adjunct devices to aid clinicians identify high-risk lesions (40). We believe that our technology offers a non-invasive, cost effective, high throughput, objective tool to identify high-risk oral LGDs several years before progression. In addition, because the brush is from the whole lesion, it prevents sampling error from the clinicians. Our test shows a high-negative predictive value of 96% that suggests that patients with low score can be subjected to less frequent biopsies and have longer follow-up intervals thus reducing the burden on our healthcare system.

Our data support the use of adjunct tools like TB and lesion brushings for DNA-ICM analysis in addition to histopathology to triage lesions at high-risk of progression. We propose that high-risk DNA brushing patients should be analyzed on a case-to-case basis in correlation with dysplasia grading, clinical risk factors, and offered a personalized follow-up plan. Such patients can be kept under closer follow-up and if needed, biopsied more frequently to detect progression early.

Oral cancer development is a complex multistep process with several molecular changes contributing to it. As reported

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

Table 4. Analysis of progression risk by combining DNA brushing classification, dysplasia grade, and lesion clinical features using a Cox regression model.

Model	*N*	Variable[b]	HR (95%CI)	*P*
1	128[a]	DNA brushing classification	5.13 (1.44–18.2)	**0.011**
		Dysplasia grade	3.88 (1.08–13.9)	**0.037**
2	128[a]	DNA brushing classification	7.32 (2.04–26.21)	**0.002**
		Ever TB positive	5.25 (1.39–19.71)	**0.014**
		Lesion area greater than 200 mm^2	4.45 (1.25–15.80)	**0.021**
3	128[a]	DNA brushing classification	8.00 (2.13–30.05)	**0.002**
		Ever TB positive	5.89 (1.53–22.69)	**0.010**
		Lesion area greater than 200 mm^2	4.87 (1.35–17.47)	**0.015**
		Dysplasia grade	4.80 (1.31–17.49)	**0.017**

[a]2 non-progressors censored cases before the earliest event in a stratum.
[b]Dysplasia grade coded as mild or moderate dysplasia; DNA brushing classification coded as high-risk or low-risk; ever TB-positive coded as Yes or No, Lesion area greater than 200 mm^2 coded as Yes or No.

by previous studies, not all lesions that progress harbor DNA aneuploidies (41). Our study showed that 19% of progressing samples did not show high-risk DNA brushing classification, which may suggest that pathways other than chromosomal instability play a role in carcinogenesis. Inability to detect DNA aneuploidy in all progressors can also be contributed to limitations of DNA-ICM technology itself, as it requires a loss or gain of at least one entire chromosome to be detected successfully. Highly sensitive techniques like comparative genomic hybridization have detected a higher percentage of aneuploid tumors in oral carcinomas in contrast with DNA-ICM or DNA-FCM (42). However, these techniques are costly and technique sensitive, and thus would have limited clinical applications.

The primary limitation of our study is the small sample size with limited progressing samples. Our findings need to be validated by a large prospective study. A number of patients were lost to follow-up at our dysplasia clinic, but access to a central biopsy service helped us monitor for progression for over 5 years following the index biopsy. Another major limitation was a significant number of samples showed low cellularity. Seventeen samples had less than 300 cells of which 2 were progressors. This can be due to the retrospective design of our study as samples dated between 2005 and 2014 were used for analysis. Variability in brushing personnel and technique may be some of the contributing factors. Limitation of obtaining less cells from oral brushings has also been reported in previously (43). Single-cell aneuploidies can easily be missed in samples with less cellularity causing false-negative results. To overcome this shortcoming, we used diploid epithelial cells to estimate CO scores in samples that lacked DNA aneuploidy or increased proliferation.

DNA-ICM can serve as a cost effective, semiautomated tool to aid clinicians in identifying high-risk oral precancers several years before progression. Because it is a non-invasive test, high-risk lesions can be subjected to repeated analysis and kept under closer observation whereas low-risk lesions can be spared from an unnecessary biopsy. A large prospective study with temporal measurements is needed to help better understand its role in progression of OPMLs.

Authors' Disclosures

L. Zhang reports grants from National Institute of Health during the conduct of the study. No disclosures were reported by the other authors.

Authors' Contributions

M. Datta: Data curation, formal analysis, funding acquisition, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **D.M. Laronde:** Conceptualization, supervision, funding acquisition, project administration, writing–review and editing. **M.P. Rosin:** Conceptualization, funding acquisition, writing–review and editing. **L. Zhang:** Conceptualization, funding acquisition, writing–review and editing. **B. Chan:** Conceptualization, writing–review and editing. **M. Guillaud:** Conceptualization, resources, formal analysis, supervision, methodology, project administration, writing–review and editing.

Acknowledgments

We thank Anita Carraro, Jagoda Korbelik, and Alan Harrison for their expert technical support. This work was supported by grants from the NIH and the National Institute of Dental and Craniofacial Research (R01DE13124 and R01DE17013). This work was also supported by the Alpha Omega Foundation of Canada (F18–05468).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 28, 2021; revised May 28, 2021; accepted July 28, 2021; published first August 10, 2021.

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024

References

1. Warnakulasuriya S, Johnson NW, Waal IVD. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007;36:575–80.
2. Warnakulasuriya S. Histological grading of oral epithelial dysplasia: revisited. J Pathol 2001;194:294–7.
3. Zhang L, Lubpairee T, Laronde DM, Rosin MP. Should severe epithelial dysplasia be treated? Oral Oncol 2016;60:125–9.
4. Poh CF. Biopsy and histopathologic diagnosis of oral premalignant and malignant lesions. J Can Dent Assoc 2008;74:283–8.
5. Karabulut A, Reibel J, Therkildsen MH, Praetorius F, Nielsen HW, Dabelsteen E. Observer variability in the histologic assessment of oral premalignant lesions. J Oral Pathol Med 1995;24:198–200.
6. Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses, and scope for improvement. J Oral Pathol Med 2008;37:127–33.
7. Mehanna HM, Rattay T, Smith J, McConkey CC. Treatment and follow-up of oral dysplasia—a systematic review and meta-analysis. Head Neck 2009;31:1600–9.

8. Holmstrup P, Dabelsteen E. Oral leukoplakia—to treat or not to treat. Oral Dis 2016;22:494–7.
9. Zhang L, Poh CF, Lam WL, Epstein JB, Cheng X, Zhang X, et al. Impact of localized treatment in reducing risk of progression of low-grade oral dysplasia: molecular evidence of incomplete resection. Oral Oncol 2001;37:505–12.
10. Rock LD, Laronde DM, Lin I, Rosin MP, Chan B, Shariati B, et al. Dysplasia should not be ignored in lichenoid mucositis. J Dent Res 2018;97:767–72.
11. Nankivell P, Mehanna H. Oral dysplasia: biomarkers, treatment, and follow-up. Curr Oncol Rep 2011;13:145–52.
12. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:612–27.
13. Smith J, Rattay T, McConkey C, Helliwell T, Mehanna H. Biomarkers in dysplasia of the oral cavity: a systematic review. Oral Oncol 2009;45: 647–53.
14. Giaretti W, Monteghirfo S, Pentenero M, Gandolfo S, Malacarne D, Castagnola P. Chromosomal instability, DNA index, dysplasia, and subsite in oral premalignancy as intermediate endpoints of risk of cancer. Cancer Epidemiol Biomark Prev 2013;22:1133–41.
15. Garner D. Clinical application of DNA ploidy to cervical cancer screening: a review. World J Clin Oncol 2014;5:931.
16. Weaver BAA, Cleveland DW. Does aneuploidy cause cancer? Curr Opin Cell Biol 2006;18:658–67.
17. Danielsen HE, Pradhan M, Novelli M. Revisiting tumour aneuploidy —the place of ploidy assessment in the molecular era. Nat Rev Clin Oncol 2016;13:291–304.
18. Faranda A, Costa A, Canova S, Abolafio G, Silvestrini R. Image and flow cytometric analyses of DNA content in human solid tumors. A comparative study. Anal Quant Cytol Histol 1997;19:338–44.
19. Mehrotra R. The role of cytology in oral lesions: a review of recent improvements. Diagn Cytopathol 2012;40:73–83.
20. Reid BJ, Levine DS, Longton G, Blount PL, Rabinovitch PS. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol 2000;95:1669–76.
21. Zink D, Fischer AH, Nickerson JA. Nuclear structure in cancer cells. Nat Rev Cancer 2004;4:677–87.
22. Hake SB, Xiao A, Allis CD. Linking the epigenetic "language" of covalent histone modifications to cancer. Br J Cancer 2004;90:761–9.
23. MacAulay C, Keyes M, Hayes M, Lo A, Wang G, Guillaud M, et al. Quantification of large scale DNA organization for predicting prostate cancer recurrence. Cytom Part J Int Soc Anal Cytol 2017;91:1164–74.
24. Guillaud M, Zhang L, Poh C, Rosin MP, MacAulay C. Potential use of quantitative tissue phenotype to predict malignant risk for oral premalignant lesions. Cancer Res 2008;68:3099–107.
25. MacAulay C, Lam S, Payne PW, LeRiche JC, Palcic B. Malignancy-associated changes in bronchial epithelial cells in biopsy specimens. Anal Quant Cytol Histol 1995;17:55–61.
26. Torres-Rendon A, Stewart R, Craig GT, Wells M, Speight PM. DNA ploidy analysis by image cytometry helps to identify oral epithelial dysplasias with a high risk of malignant progression. Oral Oncol 2009; 45:468–73.
27. Bradley G, Odell EW, Raphael S, Ho J, Le LW, Benchimol S, et al. Abnormal DNA content in oral epithelial dysplasia is associated with increased risk of progression to carcinoma. Br J Cancer 2010;103: 1432–42.
28. Bremmer JF, Brakenhoff RH, Broeckaert MAM, Beliën JAM, Leemans CR, Bloemena E, et al. Prognostic value of DNA ploidy status in patients with oral leukoplakia. Oral Oncol 2011;47:956–60.
29. Siebers TJH, Bergshoeff VE, Otte-Höller I, Kremer B, Speel EJM, van der Laak JAWM, et al. Chromosome instability predicts the progression of premalignant oral lesions. Oral Oncol 2013;49:1121–8.
30. Sperandio M, Brown AL, Lock C, Morgan PR, Coupland VH, Madden PB, et al. Predictive value of dysplasia grading and DNA ploidy in malignant transformation of oral potentially malignant disorders. Cancer Prev Res 2013;6:822–31.
31. Zaini ZM, McParland H, Møller H, Husband K, Odell EW. Predicting malignant progression in clinically high-risk lesions by DNA ploidy analysis and dysplasia grading. Sci Rep 2018;8:15874.
32. Sathasivam HP, Nayar D, Sloan P, Thomson PJ, Odell EW, Robinson M. Dysplasia and DNA ploidy to prognosticate clinical outcome in oral potentially malignant disorders. J Oral Pathol Med 2021;50:200–9.
33. MacAulay CE, Poh CF, Guillaud M, Williams PM, Laronde DM, Zhang L, et al. High-throughput image cytometry for detection of suspicious lesions in the oral cavity. J Biomed Opt 2012;17:086004.
34. Chiu D, Guillaud M, Cox D, Follen M, MacAulay C. Quality assurance system using statistical process control: an implementation for image cytometry. Anal Cell Pathol 2004;26:101–17.
35. Haroske G, Baak JP, Danielsen H, Giroud F, Gschwendtner A, Oberholzer M, et al. Fourth updated ESACP consensus report on diagnostic DNA image cytometry. Anal Cell Pathol 2001;23:89–95.
36. Jabalee J, Carraro A, Ng T, Prisman E, Garnis C, Guillaud M. Identification of malignancy-associated changes in histologically normal tumor-adjacent epithelium of patients with HPV-positive oropharyngeal cancer. Anal Cell Pathol 2018;2018:1607814.
37. Parfenova E, Liu KYP, Harrison A, MacAulay C, Guillaud M, Poh CF. An improved algorithm using a Health Canada-approved DNA-image cytometry system for non-invasive screening of high-grade oral lesions. J Oral Pathol Med 2021;50:502–9.
38. Scheurer ME, Guillaud M, Tortolero-Luna G, McAulay C, Follen M, Adler-Storthz K. Human papillomavirus-related cellular changes measured by cytometric analysis of DNA ploidy and chromatin texture. Cytometry B Clin Cytom 2007;72B:324–31.
39. Alaizari NA, Sperandio M, Odell EW, Peruzzo D, Al-Maweri SA. Meta-analysis of the predictive value of DNA aneuploidy in malignant transformation of oral potentially malignant disorders. J Oral Pathol Med 2018;47:97–103.
40. Chaturvedi AK, Udaltsova N, Engels EA, Katzel JA, Yanik EL, Katki HA, et al. Oral leukoplakia and risk of progression to oral cancer: a population-based cohort study. J Natl Cancer Inst 2020;112:1047–54.
41. Diwakar N, Sperandio M, Sherriff M, Brown A, Odell EW. Heterogeneity, histological features and DNA ploidy in oral carcinoma by image-based analysis. Oral Oncol 2005;41:416–22.
42. Okafuji M, Ita M, Oga A, Hayatsu Y, Matsuo A, Shinzato Y, et al. The relationship of genetic aberrations detected by comparative genomic hybridization to DNA ploidy and tumor size in human oral squamous cell carcinomas. J Oral Pathol Med 2000;29:226–31.
43. Datta M, Laronde D, Palcic B, Guillaud M. The role of DNA image cytometry in screening oral potentially malignant lesions using brushings: a systematic review. Oral Oncol 2019;96:51–9.

Downloaded from http://aacrjournals.org/cancerpreventionresearch/article-pdf/14/12/1111/3017220/1111.pdf by guest on 19 July 2024