

## Research Article

**Predictive Value of Dysplasia Grading and DNA Ploidy in Malignant Transformation of Oral Potentially Malignant Disorders** Marcelo Sperandio<sup>1</sup>, Amy L. Brown<sup>1</sup>, Claire Lock<sup>2</sup>, Peter R. Morgan<sup>1,2,5</sup>, Victoria H. Coupland<sup>3</sup>, Peter B. Madden<sup>3</sup>, Saman Warnakulasuriya<sup>4,5</sup>, Henrik Møller<sup>3</sup>, and Edward W. Odell<sup>1,3,5</sup>**Abstract**

Dysplasia grading is widely used to assess risk of transformation in oral potentially malignant disorders despite limited data on predictive value. DNA ploidy analysis has been proposed as an alternative. This study examines the prognostic value for both tests used in a routine diagnostic setting to inform clinical management. A retrospective study of conventional dysplasia grading was conducted on 1,401 patients. DNA ploidy analysis was conducted on a subset of 273 patients and results correlated with clinical information, pathologic diagnosis, and outcome over 5 to 15 years.

Malignant transformation occurred in 32 of 273 patients (12%) and, of these, 20 (63%) of preexisting index lesions were aneuploid. Of 241 patients not developing carcinoma, only 39 (16%) of index lesions were aneuploid. Epithelial dysplasia correlated with DNA ploidy status ( $P < 0.001$ ). The overall positive predictive value for malignant transformation by DNA aneuploidy was 38.5% (sensitivity 65.2% and specificity 75%) and by severe dysplasia grade 39.5% (sensitivity 30% and specificity 98%). DNA diploid and tetraploid status had negative predictive value of 90% to 96%. Combining DNA ploidy analysis with dysplasia grading gives a higher predictive value than either technique alone.

Each of three traditional dysplasia grades predicts a significantly different risk of carcinoma development and time to transformation. DNA ploidy analysis had equivalent predictive value and also detected additional risk lesions in the absence of dysplasia. *Cancer Prev Res*; 6(8); 822–31. ©2013 AACR.

**Introduction**

Some oral and oropharyngeal squamous cell carcinomas (SCC) arise in precursor lesions, called oral potentially malignant disorders (OPMD; ref. 1) and histologic assessment of dysplasia is currently the universally applied predictive test for risk of their malignant transformation. SCC develops in 6% to 20% of dysplastic lesions but dysplasia grading is not an accurate predictor. Most OPMD remain unchanged or regress (2) and dysplasia grading has been criticized as subjective, poorly reproducible, and inadequate for clinical management (3–5). There is a need for better predictive markers of transformation to guide treatment (6).

Chromosomal instability and DNA aneuploidy are fundamental to malignancy (7). Preneoplastic oral epithelium is usually aneuploid (8–10) and DNA aneuploidy indicates risk of malignant transformation (11). These changes can be measured using karyometry, flow cytometry (12), and image analysis (13) and more subtle loss of heterozygosity by molecular methods (14).

Image-based DNA ploidy analysis is predictive in cross-sectional (9, 15) and case-control studies (16, 17) of oral dysplastic lesions. Data from a flawed retrospective study were retracted (18) and the predictive value of ploidy analysis remains unknown in a routine diagnostic setting.

Surprisingly, despite its widespread routine use, histologic assessment of grade of dysplasia in OPMD also has no defined predictive value in clinical series, either for overall risk or time to transformation (4, 5, 19) as outlined in our review (20). Its value needs to be defined in a longitudinal series of diagnostic relevance, including clinically indeterminate and borderline lesions and those with reactive atypia, such as candidosis and frictional keratosis that are frequently confused with OPMDs clinically.

The aim of this study was to determine the positive and negative predictive values of image-based DNA ploidy analysis and dysplasia grading in predicting malignant

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**Table 1.** Numbers of patients in the study population and subgroups for the DNA ploidy analysis and dysplasia grading analysis

Total patients meeting inclusion criteria		2,310	
Excluded for lack of follow up data		720 (31.2%)	
Excluded for dysplasia or other diagnosis before 1990		146 (6.3%)	
Exclusions for minor reasons (see text)		43 (1.9%)	
Remaining patients divided into 2 study populations on basis of sufficient material for DNA ploidy analysis	Total patients in DNA ploidy analysis	Total patients in dysplasia analysis but not in DNA ploidy analysis	Overall total patients in analysis
	273	1,128	1,401
Number developing squamous carcinoma	32	17	49
Number of patients whose carcinoma developed within 6 months of index biopsy	17	5	22
Total number of patients remaining after exclusion of early transformation	256	1,123	1,379
Number of patients whose carcinoma developed more than 6 months after index biopsy	15	12	27

transformation of oral epithelium when used in the context of a routine diagnostic biopsy service serving specialist clinicians.

## Materials and Methods

### Sample selection

The study sample was defined clinically by suspicion of a risk lesion and, if no risk had been considered clinically, by pathologic diagnosis of dysplasia. The sample was designed to include all submitted biopsy specimens for which the pathology report would be expected to provide information on the possible risk of malignant transformation. The sample was, therefore, deliberately diverse and designed to match cases on which diagnostic DNA ploidy analysis might be requested by clinicians or histopathologists. Pathology reports and original biopsy request forms for all specimens submitted to the Department of Oral Pathology, King's College London from 1990 to 1999 were searched both electronically in laboratory information systems and by hand. Those that indicated a mucosal lesion with clinical suspicion of risk of transformation were selected, either because this suspicion was made explicitly by the submitting clinician, or by identifying key terms "(idiopathic) white patch" or "leukoplakia," "red patch" or "erythroplakia," "smoker's keratosis," "chronic hyperplastic candidosis," "oral submucous fibrosis," and "pre-malignancy." All cases that showed any degree of dysplasia histologically were also selected, regardless of clinical presentation or clinical risk assessment but clinically evident carcinomas and dysplastic lesions concurrent with carcinoma were excluded. The series included many cases of lichen planus and lichenoid reaction where these met the above-mentioned criteria. Eighty normal/hyperplastic mucosal samples acted as controls. Exclusion criteria were an oral biopsy

before 1990, previous oral carcinoma, insufficient sample for analysis, or lack of demographic information to obtain follow up data. Patients overall had a mean age of 54 years (SD = 13.9 years) and 38% were male. In the ploidy analysis subgroup, the mean age was 55 years (SD 13.8 years), and not statistically different from the overall group, and 54% were male. Patient selection and numbers in each subgroup are shown in Table 1.

Death (cause, date) and cancer registration (type, site, date) data were obtained from the Thames Cancer Registry and the UK Office for National Statistics (ONS). The follow up period ranged from 5 to 15 years (mean = 9.3 years, SD = 3.6 years).

### Dysplasia grading

Dysplasia grading was conducted by consensus of 2 pathologists (PRM and EWO) using WHO criteria current at time of diagnosis, with increased emphasis on architectural features, similar to subsequent and current criteria (21) but with less emphasis on thirds of epithelial thickness involved. The diagnoses for all biopsies were the original reported diagnoses and the consensus grading had been conducted prospectively for future research. Dysplasia grading was blind to the ploidy results. Patient numbers in the dysplasia analysis subgroup are shown in Table 1.

### DNA ploidy sample preparation

Patient numbers in the DNA ploidy analysis subgroup are shown in Table 1.

Epithelium for analysis, including all dysplastic areas present, was selected on hematoxylin and eosin stained sections. Areas of interest were coarsely microdissected by scoring the block surface and selectively sampled from the wax block as multiple 50  $\mu$ m sections (the number to

provide equivalent to approximately 50 mm epithelial length), deparaffinized in xylene, rehydrated in ethanols to PBS, and incubated in 2 mL 0.05% protease type XXIV (Sigma) at 37°C for 90 minutes with shaking. Released nuclei were filtered through 60- $\mu$ m nylon mesh (Millipore NY6000010), washed, and centrifuged to dispersed monolayers (Cytospin 4; Shandon).

### Image-based ploidy analysis

Nuclear monolayers were stained with Feulgen-Schiff and nuclear DNA content was measured using a Fairfield ploidy system (Medical Solutions), an automated scanning Zeiss Axioplan II microscope (Zeiss), black and white digital camera (Hamamatsu Photonics, model C4742-95), and a 546 nm green barrier filter giving an estimated resolution of 170 nm per pixel in a 1,024  $\times$  1,024 pixel field with 10-bit gray-scale resolution.

### DNA ploidy diagnostic criteria

Lymphocyte, epithelial, and fibroblast nuclei were classified by the software using a custom rules file previously defined on normal and dysplastic mucosa samples of similar age and processing to the study samples. All classifications were reviewed by a diagnostic histopathologist (EWO) before final diagnosis. A lesion was classified as DNA diploid if epithelial nuclei formed one peak at 2c and if the number of nuclei at any 4c peak did not exceed 10% of the total epithelial nuclei. Any sample with aneuploid peaks (outside diploid index of 1.8–2.2c and 3.6–4.4c) containing more than 10% of the epithelial nuclei or with more than 1% of epithelial nuclei above 5c was classified as DNA aneuploid. DNA tetraploid samples were an intermediate group with a 4c peak exceeding 10% of the total epithelial nuclei but no other abnormality. True chromosomal tetraploid samples (with an epithelial 4c peak exceeding 10% of the 2c peak and additional S phase events extending to an 8c peak) were not found except as part of a multi-aneuploid profile. A minimum of 300 epithelial nuclei were assessed and samples with a diploid peak coefficient of variation of greater than 5% were excluded from the analysis, unless below 6% and grossly multiploid and with a high 5c exceeding rate (16 analyses). As far as possible using the Fairfield system, criteria met those recommended by the ESACP consensus report (22).

### Statistical analyses

The unit of analysis for malignant transformation was the patient. When more than one biopsy per patient existed, the earliest specimen showing the most abnormal result was used as the index lesion for analysis ("first-worst" analysis). When no dysplasia or DNA ploidy abnormality was found, the earliest lesion was taken as the index lesion. Patients were analyzed as 2 groups, those who developed a carcinoma at any time and those who developed carcinoma within 6 months of the index specimen (Table 1). The latter were analyzed as a separate group because it is possible that carcinoma had been present at the time of biopsy but not diagnosed because of clinical sampling error. Other asso-

ciations between dysplasia and clinical parameters and between dysplasia and DNA ploidy status were analyzed by individual lesion (sample).

Positive and negative predictive values, sensitivity, and specificity were calculated directly taking malignant transformation as the reference. The association between DNA ploidy diagnoses and between dysplasia grades were calculated using Pearson  $\chi^2$  test and Cox HRs. Malignant transformation was analyzed by Kaplan–Meier survival curve analysis and time to transformation by dysplasia grade and ploidy diagnosis were compared using the log rank (Mantel–Cox) test. Malignant transformation was the event of interest with censoring events being death or end of follow-up, the latter a minimum period of 5 years follow-up since the last biopsy specimen taken. The total population and DNA ploidy subset were analyzed as independent groups and no analyses compared the 2 groups.

The study was approved by the Guy's Hospital Research Ethics Committee and the use of material and data by the UK Patient Information Advisory Group [reference PIAG 4-09(f)2003].

### Results

The patient population, subgroups analyzed, and numbers who developed oral squamous carcinoma are shown in Table 1. Dysplasia results and follow up data on malignant transformation were available from 1,401 patients. Within this population, a subset of 273 patients was included in the DNA ploidy analysis. A total of 49 of the 1,401 patients developed carcinoma of which 32 were included in the DNA ploidy analysis. Of the patients who developed carcinoma, 22 transformed within 6 months of the index lesion and 17 of these were included in the DNA ploidy analysis.

A total of 1,838 lesions arising in these 1,401 patients were analyzed for dysplasia grade and 1,401 index lesions were selected as above for transformation analysis. A total of 729 biopsy samples were analyzed for DNA ploidy and 273 index lesions were identified using the criteria above for transformation analysis. The sites of index biopsy of samples for DNA ploidy analysis included 76 (30%) buccal mucosa, 39 (15%) lateral tongue, 26 (10%) floor of mouth, the remainder were from diverse oral sites ( $n = 273$ ). The grades of dysplasia found in the whole group of 729 lesions analyzed of the DNA ploidy analysis cohort are shown in Table 2.

### Dysplasia and DNA ploidy

All control samples were DNA diploid. Of the 729 lesions tested overall, 170 (23.3%) were aneuploid, 98 (13.4%) tetraploid, and 461 (63.3%) diploid ( $n = 729$ ; Table 2). Of the index lesions, 59 (21.6%) were aneuploid, 53 (19.4%) were tetraploid, and 161 (59%) were diploid and of this group, 29 (10.6%) showed severe dysplasia, 69 (25.3%) moderate dysplasia, 103 (37.7%) mild dysplasia, and 72 (26.4%) were nondysplastic ( $n = 273$ ). No nondysplastic lesions showed a DNA ploidy abnormality.

**Table 2.** Frequency distribution of DNA ploidy diagnosis by degree of dysplasia in all lesions from the DNA ploidy subgroup, including index and nonindex lesions

DNA ploidy diagnosis	Dysplasia grade				Total <sup>a</sup> n (%) <sup>b</sup>
	None n (%) <sup>b</sup>	Mild n (%) <sup>b</sup>	Moderate n (%) <sup>b</sup>	Severe n (%) <sup>b</sup>	
Diploid	241 (52)	93 (20)	80 (17)	47 (10)	461 (100)
Tetraploid	53 (54)	17 (17)	14 (14)	14 (14)	98 (100)
Aneuploid	31 (18)	18 (11)	72 (42)	49 (29)	170 (100)
Total	325 (45)	128 (18)	166 (23)	110 (15)	729 (100)

<sup>a</sup>This analysis includes 729 individual lesions of all types for which a valid result for DNA ploidy and dysplasia grade were available, and excludes those for which no follow-up data were available. This set of individual lesions includes both index and nonindex lesions and is not identifiable from Table 1, Table 4, or Fig. 2 (which show patient-based data). Nondysplastic lesions had all met the inclusion criteria and were considered suspicious on clinical grounds.

<sup>b</sup>Percentage of total lesions with a ploidy diagnosis.

There is a statistically significant association between dysplasia grade and DNA ploidy, Pearson  $\chi^2 = 107.46$ ,  $df = 6$ ,  $P < 0.001$ .

Dysplasia grade and DNA ploidy status were significantly associated with one another ( $\chi^2 = 107.46$ ,  $df = 6$ ,  $P < 0.001$ ). As shown in Table 2, most diploid and tetraploid lesions were nondysplastic or mildly dysplastic and most aneuploid lesions were moderately or severely dysplastic. A total of 31 of 325 lesions without dysplasia were aneuploid. Both markers were equally well associated with transformation overall, with no statistical differences between transformation survival curves (Fig. 1).

DNA ploidy diagnoses for the index lesions classified by clinical presentation are shown in Table 3, excluding those

patients who developed carcinoma within 6 months to avoid possible undetected carcinomas. The majority were white lesions and the majority of abnormal DNA ploidy results were obtained from white lesions. Detailed analysis of the clinical risk factors in this population has been published elsewhere (23).

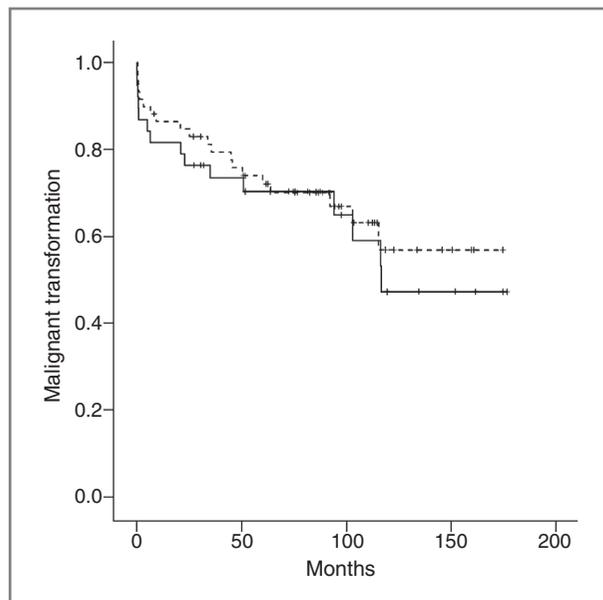
#### Dysplasia grading and malignant transformation

Excluding patients who developed carcinoma within 6 months of the index biopsy, a total of 27 patients developed carcinoma (from total  $n = 1,379$ ; Table 1). Four of 103 (4%), 7 of 69 (10%), and 6 of 29 (21%) patients with mild, moderate, or severe dysplasia respectively, in index lesions developed carcinoma and 10 of 1,178 (0.85%) patients with nondysplastic index lesions (and therefore with no dysplasia in any other of any multiple/sequential biopsies) developed carcinoma (data not included in Table 1).

Including patients with transformation within 6 months (total  $n = 1,401$ ), 14 of 1,182 patients with nondysplastic lesions, 6 of 105 (6%), 14 of 76 (18%), and 15 of 38 (39%) patients with mild, moderate, or severely dysplastic index lesions respectively, underwent malignant transformation to squamous cell carcinoma (total 49; Table 1).

In the group that excluded those patients who underwent malignant transformation within 6 months ( $n = 1,379$ ), the risk of a mild dysplasia becoming malignant was 5.3 times higher than for a nondysplastic lesion (95% CI, 1.6–16.8). The risk for lesions with moderate and severe dysplasia was 12.8 (95% CI, 4.9–33.7) and 29.9 (95% CI, 10.8–82.5) times higher, respectively. All HRs were highly significant ( $P \leq 0.005$ ) and patients with higher dysplasia grades developed carcinoma more rapidly (log rank:  $\chi^2 = 100.21$ ,  $df = 3$ ,  $P < 0.0001$ ).

When patients with transformation within 6 months of the index biopsy were included ( $n = 1,401$ ), the risk of a mild dysplasia becoming malignant was similar at 5.3 (95% CI, 2.0–13.8) times higher than for a nondysplastic



**Figure 1.** Kaplan–Meier survival curves for risk of malignant transformation in patients with either severe dysplasia (solid line) or DNA aneuploidy (dashed line) in the index lesion. There is no significant difference between the 2 predictors of transformation, log rank:  $\chi^2 = 0.259$ ,  $df = 1$ ,  $P = 0.61$ .

**Table 3.** DNA ploidy status of index lesions by clinical diagnosis for patients with transformation after 6 months

Diagnosis	Number diploid	Number tetraploid	Number aneuploid	Total <sup>a</sup>
Leukoplakia including idiopathic/smoker's keratosis	116 (45.3)	39 (15.2)	34 (13.3)	189 (73.8)
Lichen planus or lichenoid reaction	19 (7.4)	4 (1.6)	0	23 (9)
Erythroplakia	1 (0.4)	0	0	1 (0.4)
Oral submucous fibrosis	3 (1.2)	0	0	3 (1.2)
Chronic hyperplastic candidosis	12 (4.7)	6 (2.3)	6 (2.3)	24 (9.4)
Actinic keratosis	1 (0.4)	0	0	1 (0.4)
Other <sup>b</sup>	7 (2.7)	2 (0.8)	6 (2.3)	15 (5.9)
Total	159 (62.1)	51 (19.9)	46 (18)	256 (100) <sup>a</sup>

NOTE: Number of lesions and percentage of total in parentheses.

<sup>a</sup> $n = (273 \text{ patients less } 17 \text{ with early transformation}) = 256$  (Table 1).<sup>b</sup>Includes chronic nonspecific ulceration, inflammation, papillary hyperplasia, etc. histologically, but considered as possible risk lesions clinically. Proliferative verrucous leukoplakia not separately identified as malignant transformation is a defining clinical characteristic.

lesion. HRs for moderate and severe dysplasia were increased to 17.5 (95% CI, 8.3–36.6) and 46.5 (95% CI, 22.4–96.5) times higher, respectively. All differences were highly significant ( $P \leq 0.001$ ). Patients with higher grades of dysplasia suffered transformation more rapidly (log rank:  $\chi^2 = 274.48$ ,  $df = 3$ ,  $P < 0.0001$ ).

**DNA ploidy analysis and malignant transformation**

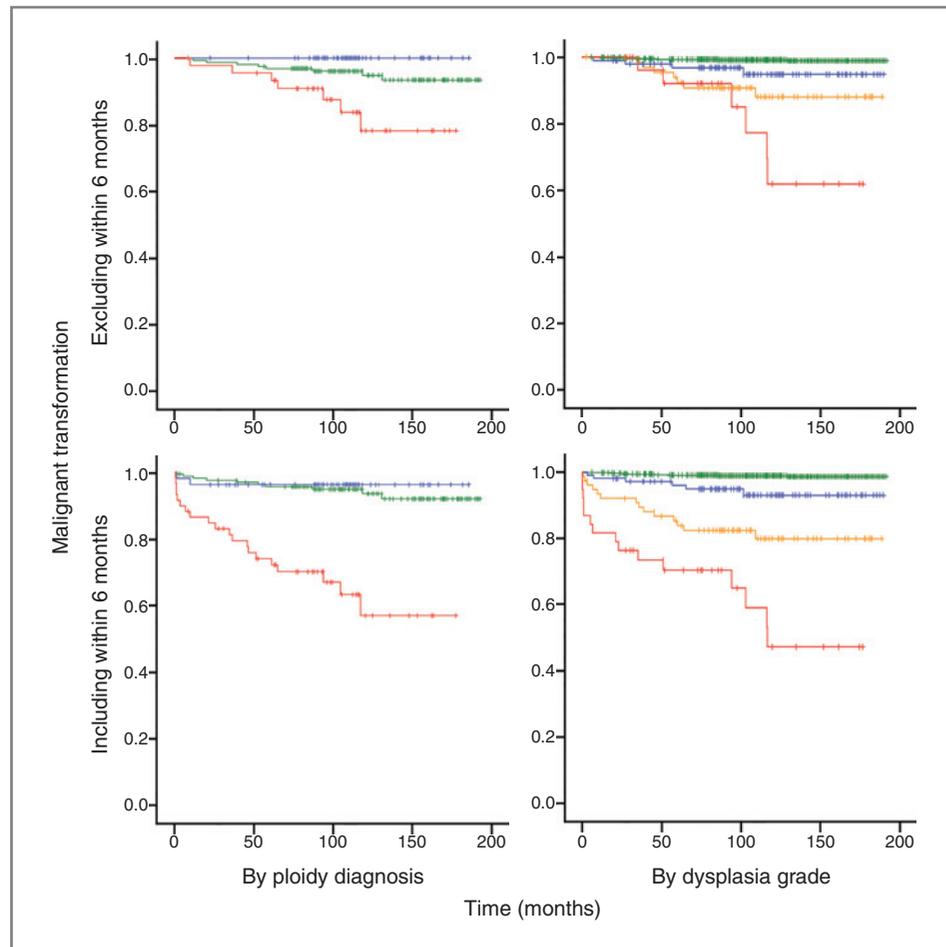
Excluding patients who developed carcinoma within 6 months of the index biopsy ( $n = 256$ ), 8 of 159 (5%) patients with diploid index lesions developed carcinoma. No transformation occurred in 51 patients with tetraploid index lesions. Seven of the 46 (15%) patients with

**Table 4.** Patient-based analysis of positive and negative predictive values of DNA ploidy and dysplasia grading for patients with and without malignant transformation

	Excluding malignant transformation within 6 months of index biopsy		Including malignant transformation within 6 months of index biopsy	
	PPV%	NPV%	PPV%	NPV%
DNA aneuploidy number of cases	$n = 256$		$n = 273$	
DNA aneuploidy	15.2		<b>34</b>	
DNA diploid or tetraploid		96.6		<b>94.4</b>
DNA aneuploidy + dysplasia number of cases	$n = 110$		$n = 119$	
DNA aneuploidy in cases with any degree of dysplasia	27.3		<b>39</b>	
DNA diploid or tetraploid in cases with any degree of dysplasia		92.2		<b>90</b>
Dysplasia number of cases	$n = 1,379$		$n = 1,401$	
Severe dysplasia	20.7	98.4	<b>39.5</b>	97.5
Moderate dysplasia	10.1	98.9	<b>18.4</b>	98.4
Moderate or severe	13.3	98.9	25.4	98.4
Mild dysplasia	3.9	99.1	<b>6</b>	98.8
No dysplasia	0.85	91.5	<b>1.1</b>	84
DNA ploidy subset number of cases	$n = 110$		$n = 119$	
Severe dysplasia in the DNA ploidy subset only	23.5	88.2	38.1	84.7
Moderate dysplasia in the DNA ploidy subset only	35	92.3	22.2	90.6
Moderate or severe dysplasia in the DNA ploidy subset only	18.9	92.3	27.3	90.6
Mild dysplasia in the DNA ploidy subset only	7.7	81.0	9.4	72.7

NOTE: Cases transforming within 6 months of the index biopsy are shown separately. Data for the cases in the DNA ploidy subset are shown for comparison. Predictive values of most significance for clinical management of an initial biopsy are shown in bold. % and number of cases from which the data are derived above.

**Figure 2.** Kaplan–Meier survival analysis for rate of malignant transformation by ploidy diagnosis (left) and dysplasia grade (right). The upper graphs exclude patients with malignant transformation within 6 months of the index biopsy and the lower graphs include all patients. Ploidy key: blue diploid, green tetraploid, red aneuploid. Dysplasia key: green nondysplastic, blue mild dysplasia, amber moderate dysplasia, red severe dysplasia. There are significant differences in transformation rate between ploidy diagnoses and dysplasia grades: ploidy excluding early transformation  $n = 256$ , log rank:  $\chi^2 = 13.46$ ,  $df = 2$ ,  $P = 0.0012$ ; ploidy including early transformation  $n = 273$ , log rank:  $\chi^2 = 44.25$ ,  $df = 2$ ,  $P < 0.0001$ ; dysplasia excluding early transformation  $n = 1,379$ , log rank:  $\chi^2 = 100.21$ ,  $df = 3$ ,  $P < 0.0001$ ; dysplasia including early transformation  $n = 1,401$ , log rank:  $\chi^2 = 274.48$ ,  $df = 3$ ,  $P < 0.0001$ . Predictive values for those groups are shown in the left side of Table 4.



aneuploid index lesions developed carcinoma. When the patients who underwent malignant transformation within 6 months of their index biopsy were included (total  $n = 273$ ), 10 of the 161 (6%) diploid, 2 of the 53 (4%) tetraploid, and 20 of the 59 (34%) aneuploid cases underwent malignant transformation.

In the group developing malignant transformation after 6 months ( $n = 256$ ), none of the patients with a tetraploid index lesion developed carcinoma and the HRs were calculated on the combined diploid/tetraploid group. Patients with aneuploid index lesions had a 5.1 (95% CI, 1.8–14.2) times higher risk of transformation than those with diploid or tetraploid lesions ( $P = 0.002$ ). Patients with aneuploid index lesions developed cancer more rapidly than those with diploid and tetraploid lesions (Fig. 2; log rank:  $\chi^2 = 13.46$ ,  $df = 2$ ,  $P = 0.0012$ ).

In the group that included transformation within 6 months of the index biopsy ( $n = 273$ ), the HR for a patient with a tetraploid index lesion was 0.7 (95% CI, 0.15–3.12) of a diploid index lesion, and there was no significant difference between these 2 groups ( $P = 0.623$ ). The risk for a patient with an aneuploid index lesion was 7.4 (95% CI, 3.4–15.9) and this increased risk was highly significant ( $P < 0.001$ ) compared to diploid and tetraploid cases.

Patients with aneuploid index lesions suffered transformation more rapidly than the other 2 groups (log rank:  $\chi^2 = 44.25$ ,  $df = 2$ ,  $P < 0.0001$ ).

Kaplan–Meier curves for malignant transformation in all groups are shown in Fig. 2. Those with aneuploid or severely dysplastic lesions had the highest rates of transformation and there were significant differences between ploidy diagnoses and dysplasia grades.

#### Predictive values for dysplasia grading with DNA ploidy analysis

Excluding cases that developed carcinoma within 6 months, diploid and tetraploid groups were not significantly different and were combined for further analysis. DNA ploidy analysis of index lesions with or without dysplasia had sensitivity of 47% and specificity of 84% ( $n = 256$ ). In lesions with all grades of dysplasia ( $n = 110$ ; Table 2), the sensitivity increased to 60% but the specificity reduced to 75%.

Including cases with transformation within 6 months of the index biopsy ( $n = 273$ ) equivalent values were sensitivity 63% and specificity 84% for dysplastic and nondysplastic index lesions and a sensitivity of 65% and specificity 75% in dysplastic lesions only ( $n = 119$ ). Predictive values are shown in Table 4.

## Discussion

The association between cell DNA content and risk of malignant transformation in oral lesions is known (8, 11) but, until now, it has not been evaluated as a diagnostic test in a clinically relevant sequential series of biopsy specimens that includes many low risk or completely benign lesions. This series suggests that approximately one third of specimens submitted for biopsy to exclude dysplasia have neither dysplasia nor a ploidy abnormality (Table 2) and therefore seem to have no significant risk of transformation. This reflects that an appropriate index of suspicion by clinical staff will always generate samples of innocuous conditions taken according to the precautionary principle. We envisage that ploidy analysis could be used on all lesions submitted with clinical suspicion of malignant potential and have selected this broad through heterogeneous sample to assess its value in this context. This has led to overrepresentation of buccal lesions in comparison with studies of leukoplakia, but we have studied the clinically appropriate sample that might be subjected to the test. It is critically important to include these low risk potentially confounding lesions in studies to determine a clinically relevant predictive value because predictive value depends on the prevalence of malignant transformation.

Image-based analysis of DNA ploidy has advantages for routine diagnosis. It uses formalin-fixed samples, allows selection of areas of interest and visual inspection of nuclei, and can be partly automated. As in flow cytometry, total DNA content rather than chromosomal ploidy status is measured.

Extraction of nuclei from mucosal epithelium is relatively difficult and many specimens were too small for extraction or yielded too few nuclei for analysis. This is the main reason that ploidy analysis was conducted on only a small subset of the overall sample, even though analysis was attempted on all samples. In our prospective analysis in progress, analysis has become more reliable and a ploidy result can be obtained from almost all specimens. Nuclei were obtained from all control material embedded for up to 12 years (data not shown), consistent with previous reports (24). Automated sorting of nuclear images was conducted using a sequential series of 12 image processing parameters defined in a separate training sample set. It proved possible to diagnose almost all monolayers and all control specimens proved diploid.

DNA ploidy was generally associated with epithelial dysplasia grade, but 10% of lesions were diploid despite being severely dysplastic and 29% were aneuploid with mild or no dysplasia, consistent with previous case-control studies (9, 16, 17, 25, 26). Only one flow cytometry (27), one image-based (15), and one whole section cytometry study disagree, the last a less accurate method. Correlation is expected, but DNA ploidy and dysplasia seem independent processes and, as shown here, chromosomal instability is not necessarily accompanied by histologically detectable dysplasia.

This study benefits from inclusion of sequential samples from patients during a long follow-up period. The first of

any series of biopsy specimens with the worst dysplasia or ploidy grade ("first worst lesion") was selected as the index lesion and this reflects how patients are managed in the clinical setting with risk based on their highest dysplasia score. In this study, 53 patients of 1,401 (4%) in the larger dysplasia population and 20 of 273 (7%) in the DNA ploidy population had 4 or more biopsies whereas more than 64% in both populations had a single biopsy. Patients with multiple biopsy specimens must be presumed to have either more extensive lesions and/or other features to suggest high-risk for malignant change. Analysis on a first sample basis would risk false negative results, and "first worst" analysis is recommended for studies of this type, rather than selecting index lesions on the basis of time or site alone.

Positive and negative predictive values depend on disease prevalence and the malignant transformation rates reported in predictive marker studies are often high, reflecting the study population (28, 29), case selection, high risk habits, and therapeutic interventions (30). A total of 49 of 1,401 (3.5%) patients in this series are known to have developed carcinoma in the follow-up period, a high proportion in terms of the natural history of the disease (2) but comparable to other hospital referral series, and the predictive values obtained are therefore those applicable to specialist hospital practice. The transformation rate was higher among patients in the DNA ploidy analysis (12%) than for those in the dysplasia analysis only (2%). This selection bias is probably explained by the need for larger samples for ploidy analysis, favoring inclusion of larger lesions and patients with multiple lesions, a higher risk population.

The 2 populations tested, the overall dysplasia set and the DNA ploidy subset, can be regarded as independent for analysis purposes. There are no analyses in which the 2 populations are compared and the smaller DNA ploidy population is not fully representative of the larger group. The definitive data on dysplasia is obtained from the larger group and for DNA ploidy from the smaller group. The higher transformation rate in the DNA ploidy subgroup noted above has had some effect on the predictive values obtained in the ploidy analysis subset and so the equivalent predictive values for dysplasia in the DNA ploidy subset are shown for comparison in the lower rows in Table 4. As can be seen, many values are similar, well within expected variation for dysplasia grading and minimal when significant dysplasia is present. Thus, the higher transformation rate noted in this smaller group has little effect on dysplasia predictive values, the main effects being seen for moderate dysplasia, the category in which there is greatest variation in predictive value in the literature (31). Nevertheless, it remains likely that the predictive value has been artificially increased by the higher transformation rate and the slightly more frequent multiple biopsy specimens in the smaller DNA ploidy study population. Larger prospective studies will be required to refine the value of the test.

The predictive value of dysplasia grading remains controversial. Some frequently cited studies (e.g., 19) that

associate transformation and dysplasia provide rates of transformation that are outliers in meta-analysis (2). Many well-designed studies report almost no value (4, 5, 32, 33) whereas others recommend combining grades to improve prediction (31). Reproducibility of grading is also problematic. Dysplasia diagnoses in this study population were all made as part of a prospective research design by consensus of 2 pathologists blind to the ploidy diagnosis, and the dysplasia arm of the study is thus essentially prospective, whereas the ploidy arm is retrospective. This large study with actuarial patient-centered analysis shows clearly that grading dysplasia into none and 3 categories is justified by outcome and that all grades of dysplasia have independent prognostic value, both for transformation and time to transformation. The predictive values for severe dysplasia of 39.5% and HR of 46.5 are high and there are significant differences between grades by outcome. These rates include early transformation and are those most applicable to clinical practice.

We have found DNA ploidy to have a high predictive value for malignant transformation, consistent with smaller case-control studies using image-based or flow cytometry (9, 16, 17). Carcinoma developed more frequently in patients with aneuploid lesions (PPV 15.2%) than in patients with diploid or tetraploid lesions (NPV 96.6%). This is consistent with most cohort studies that reported DNA aneuploidy to be more frequent than diploidy in OPMD that later developed carcinoma (9, 25, 26).

Combining dysplasia grading with DNA ploidy provides a higher predictive value than for either technique alone, reflecting that some nondysplastic lesions are aneuploid and some severely dysplastic lesions are diploid. Ploidy analysis would seem to have particular value to detect aneuploidy in these otherwise underrated lesions. It is unclear whether these lesions might account entirely for the poor predictive value of dysplasia in previous studies, a failure more often blamed on lack of reproducibility or the subjective nature of dysplasia interpretation. This study shows that there are oral potentially malignant disorders with chromosomal instability whose gross genetic changes do not manifest as dysplasia histologically but that are still at risk of transformation.

Most studies of OPMDs exclude malignant transformation within 6 months of the index biopsy, reasoning that carcinoma had already been present but was inadequately sampled or diagnosed. This exclusion is necessary in longitudinal studies designed to define the natural history of OPMDs but would be inappropriate in a study designed to define the value of a diagnostic test. Including early transformation is clinically relevant and here both analyses of transformation have been presented. We have shown that in a clinical context the presence of moderate, severe dysplasia, or DNA aneuploidy indicate an immediate risk of carcinoma and that dysplasia grade and DNA ploidy both provide information on time to transformation. The highest predictive values were obtained for DNA ploidy analysis in dysplastic lesions. The positive predictive value of aneuploidy in dysplastic

lesions, 39%, was nearly double than that for severe dysplasia alone, 20.7%.

The DNA ploidy diagnostic categories used in this study are derived from accepted criteria for cytometry in sections, flow cytometry, and other techniques (22) but they have not been validated for clinical reporting in OPMD. Defining aneuploidy on histograms remains partly subjective. In this study DNA aneuploidy was readily detected. No peridiploid peaks, shoulders, or minor peaks complicated diagnosis and almost all aneuploid histograms had obvious peaks between 2c and 4c and with a 5c exceeding rate of 1% or more. A small proportion of histograms were borderline for aneuploidy and refinement of the diagnostic criteria for predictive value might prove valuable as several patterns of DNA aneuploidy were detected.

No site-specific analysis has been undertaken. Almost all of the biopsy samples had been submitted on forms with a mouth map diagram indicating the site relatively accurately. However, in only a handful of cases was it possible to determine that the biopsy site was within 1 cm of the subsequent carcinoma, too few for analysis. Conversely, in many cases it was notable that the biopsy site was relatively remote, indicating that both dysplasia grade and DNA ploidy analysis should be regarded as indicators of field change and a risk of malignant transformation throughout the mouth and probably elsewhere in the upper aerodigestive tract.

The need for effective predictive tests in OPMD is well recognized and DNA ploidy is a relatively cheap semiautomated process that is readily applied in the routine diagnostic setting. Accurate identification of those at risk, which seems to require both DNA ploidy analysis and conventional dysplasia grading, will allow more aggressive intervention in a small number of high-risk patients while allowing those at minimal risk to avoid the associated morbidity of treatment. It also has the potential to reduce costs by avoiding needless long-term follow-up for low-risk patients in secondary care. Significant savings might be possible, and a formal health economic analysis would seem warranted. It has not been possible to conduct any clinical cost analysis in our study because we are unaware whether patients with single biopsy samples remained under review in secondary care or were discharged to primary care for follow up. Lesion mapping, confirmation of excision of lesions, risk stratification in clinical trials, and patient selection for new treatments such as chemoprevention are also areas in which this type of predictive test may prove valuable.

In conclusion, we have shown in a large series that both dysplasia grading and DNA ploidy analysis have a high predictive value for carcinoma development in OPMDs and that higher dysplasia grades indicate higher degrees of risk and shorter time to transformation. The risk is patient specific not site or lesion based and persists over at least 15 years. DNA ploidy and dysplasia are correlated but do not detect the same risk population. Their combined use provides the highest predictive values and further studies should derive algorithms for risk assessment of OPMD. These predictive values have been determined in a hospital

referral patient series and are based on application of the techniques to all lesions considered to carry a risk clinically. Our results support the use of DNA ploidy in routine diagnostic use but cannot be interpreted to support its use as a screening technique or in patients without clinical risk lesions. Dysplasia grading has been criticized as subjective and poorly reproducible, but it works well and should be refined. DNA ploidy analysis is objective and merits assessment in prospective trials with dysplasia grading for clinical decision making and in stratification for clinical trials.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Sperandio, C. Lock, P.R. Morgan, S. Warnakulasuriya, E.W. Odell

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Sperandio, A.L. Brown, P.R. Morgan, V.H. Coupland, P.B. Madden, H. Moller, E.W. Odell

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# Cancer Prevention Research

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