# DNA aneuploidy and tissue architecture in oral potentially malignant disorders with epithelial dysplasia assessed by a 10 locus FISH panel

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**Abstract.** Subjectivity in oral dysplasia grading has prompted evaluation of molecular-based tests to predict malignant transformation. Aneuploidy detected by DNA image-based cytometry (ICM) is currently the best predictor but fails to detect certain high risk lesions. A novel multiplex fluorescence *in situ* hybridization (FISH) panel was used to explore possible explanations by detecting aneuploidy at the single cell level. FISH was compared to reference standard DNA ICM in 19 oral lesions with epithelial dysplasia and used to characterize the cellular architecture. Copy number variation at 3q28, 7p11.2, 8q24.3, 11q13.3 and 20q13.12 and matched chromosome specific loci were assessed by dual-color FISH to assess numerical and spatial patterns of copy number increase and gene amplification. FISH revealed wide variation in copy number at different loci. Only low level copy number gain was present and often in only a small proportion of cells, although usually with all or all but one locus (9/12). Four cases showed gene amplification, one at two loci. Some probes revealed an internal presumed clonal structure within lesions not apparent in routine histological examination. Both methods produced similar diagnostic results with concordance in detection of aneuploidy by both methods in 17 out of 19 samples (89%). We have shown that oral dysplastic lesions may contain very few aneuploid cells at a cellular level, high copy number gain is rare and changes appear to arise from large chromosomal fragment duplications. Single stem lines are relatively homogeneous for loci with copy number gain but there is a subclonal structure revealed by gene amplification in some lesions.

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*Abbreviations:* CGH, comparative genomic hybridization; CI, chromosomal instability; FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate; ICM, image-based cytometry; OED, oral epithelial dysplasia; OPMD, oral potentially malignant disorders; OSCC, oral squamous cell carcinoma; SNP, single nucleotide polymorphism; 3SD, three-times the standard deviation; *CCND1*, cyclin D1; *TP63*, tumor protein P63; *EGFR*, epidermal growth factor receptor; *PTK2*, protein tyrosine kinase 2; *MMP9*, matrix metalloproteinase-9

*Key words:* oral potentially malignant disorders, oral epithelial dysplasia, DNA image cytometry, ploidy analysis, *in situ* hybridization, leukoplakia

## Introduction

Oral squamous cell carcinoma (OSCC) results in significant morbidity and mortality. Early diagnosis is the key to prolonged survival (1, 2) and detection at a preinvasive stage is a clinical priority. Many oral potentially malignant disorders (OPMD) that predispose to oral carcinoma are recognized (3) and biopsy for detection and grading of dysplasia is the standard of care to assess the risk of malignant transformation. Unfortunately, the grading of oral epithelial dysplasia (OED) has proven to be a poor predictor of malignant transformation in several large studies (4-6). Dysplasia may regress (7-9) and non-dysplastic lesions may transform (4,10,11).

We (12,13) and others (14) have shown that DNA ploidy analysis assessed by DNA image cytometry (ICM) can predict malignant transformation in OPMD. This matches its clinical utility in a range of other human precancers (15) and in light of the fact that chromosomal instability (CI) is a hallmark of cancer (16). Alternative techniques to detect chromosomal instability in OPMD include comparative genomic hybridization (CGH) (17,18), single nucleotide polymorphism (SNP) analysis (19) and fluorescence *in situ* hybridization (FISH) (20).

The molecular changes of oral potentially malignant diseases are well described at the level of whole tissue (21). However, there is almost no data describing the clonal architecture of oral dysplasia, which can only be revealed by a single cell technique because of the relatively small size of lesions. The clonal structure of dysplasia in other sites such as colon has been well defined and has implications for the diagnosis and management of field change. The aims of this investigation were to determine the number and tissue organization of aneuploid cells in oral dysplasia, the number of loci affected in different cells and to compare findings using a FISH marker panel with image-based DNA ploidy analysis.

## Materials and methods

*Tissue samples*. Oral biopsy samples from 20 patients with epithelial dysplasia were retrieved from diagnostic archives of the Head and Neck Pathology Service at Kings College London/Guy's Hospital, London, UK. Patient consent procedures for use of tissue and information were approved by the UK Patient Information Advisory Group. DNA ploidy status was defined by ICM of dispersed Feulgen-stained epithelial nuclei monolayers prepared from formalin-fixed paraffin-embedded tissue after microdissection as previously described (12). Briefly, 50 $\mu$m thick paraffin sections were dewaxed in xylene and rehydrated in alcohols. Nuclei were extracted by incubation in 0.05% protease type XXIV (Sigma-Aldrich; Merck KGaA) at 37˚C for 90 min with shaking and cytospin monolayers were stained with Fuelgen-PAS. Monolayers were analyzed (ZMZ) on a semi-automated ploidy analyzer (Room4 Group Ltd., UK) and all images were reviewed by a pathologist (EWO). ICM DNA ploidy result was the reference test for ploidy status. Dysplasia was graded by agreement of two histopathologists. Cases were selected to provide a range of dysplasia grades and ploidy status; 7 with mild, 10 with moderate and 3 with severe dysplasia, of which 2 mild, 4 moderate and 1 severe case were diploid and the remainder aneuploid on ICM DNA ploidy.

*Fluorescence in situ hybridization*

*DNA probes*. Sections from each sample were subjected to five separate duplex FISH hybridizations to one of each of five chromosomal loci known to be frequently duplicated in OPMD. For each region, a well characterized gene was selected as a marker for the region rather than any putative role in oncogenesis. The regions were 3q28 (*TP63*), 7p11.2 (*EGFR*), 8q24.3 (*PTK2*), 11q13.3 (*CCND1*) and 20q13.12 (*MMP9*) labelled with Texas Red and a complementary centromeric probe labelled with fluorescein isothiocyanate (FITC) or, in the case of chromosome 20, a telomeric probe. The chromosome 7 probe pair was commercially available and the remainder were custom designed (Cytocell, UK) (sequences are provided in Table SI). Correct localization of novel probes was confirmed on metaphase preparations.

*Procedure*. FISH was performed according to the manufacturer's protocol (Cytocell, UK), with minor modifications, on 5-$\mu$m paraffin-embedded tissue sections mounted on charged slides. In brief, after dewaxing and rehydration, tissues were pre-treated with Aquarius LPS100 enzyme solution (Cytocell, UK), dehydrated and incubated with 10 $\mu$l of premixed probe solution sealed under a coverslip, denatured at 85˚C for 5 min and hybridized at 37˚C for 20 h. Excess probe was removed by washing for 2 min in 0.4X SSC stringent wash (pH 7.0) at 72˚C, 2X SSC/0.05% Tween and finally PBS, all at room temperature. Slides were air-dried and mounted with 10 $\mu$l of DAPI nuclear counterstain containing premixed antifade (Cytocell). Slides were stored in the dark at 4˚C until image capture.

*Scoring and analysis*. All scoring was performed blinded to the DNA ploidy status and dysplasia grade. FISH signals in 47 independent tissue areas were visualized using an Olympus BX61 microscope with appropriate filters. Preliminary counts identified the epithelium with the highest signal counts independently for each probe pair. In all cases this area was the same area displaying the highest grade of dysplasia. Depending on the size of the tissue, up to five evenly spaced areas were selected for copy number assessment.

Red and green FISH signals were counted in 200 discrete non-overlapping nuclei in continuous sequences of basal and para-basal cells up to five cells thick superficial to the basement membrane in composite photomicrographs captured at x600 magnification with an Olympus XM10 camera and Image Cell software (Olympus Corp.). Blinded duplicate counts were performed to calculate interobserver error.

Nuclei in the underlying connective tissue acted as a methodological and diploid control. All areas assessed were from within the area of epithelium that had been subjected to DNA ICM.

*Threshold detection and statistical analysis*. Cut-off FISH signal counts to define aneuploidy were calculated for each probe using two standard methods; the mean percentage of control diploid cells with signal count of three or four plus three-times standard deviation (mean + 3SD) (22) and receiver operating characteristic (ROC) analysis. Nuclei showing gene amplification, defined as tight clusters of numerous overlapping signals that could not be counted, were classified as DNA aneuploid without numerical analysis.

SPSS version 21 (IBM Corp.) was used to calculate the mean percentage of nuclei with each copy number for each target and to perform ROC. Power calculation and probability estimates for ROC analysis were performed using MedCalc version 15.6.1 (MedCalc Ostend). The agreement between DNA ICM ploidy and FISH ploidy results was determined by calculating the $\kappa$-statistic.

## Results

Of the 20 samples included in the study, 8 were from males. The average age of the patients was 62 years (age range, 31-90 years). Four of the patients were smokers, 9 had no history of smoking while the smoking history of the remainder was unknown. The sites of biopsy were lateral tongue (n=10), ventral tongue (n=1), tongue (n=1), buccal mucosa (n=3), floor of the mouth (n=3), gingiva (n=1) and soft palate (n=1).

A total of 47 tissue areas were evaluated by FISH from the 19 samples (3 cases with one area assessed, 8 cases with

Table I. FISH ploidy status for each probe in each tissue sample.

| | Chromosome locus | | | | | | | | | | Total probes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample number | Cen3 | 3q28 | Cen7 | 7p11.2 | Cen8 | 8q24.3 | Cen11 | 11q13.3 | Tel20p | 20q13.12 | D | A | Overall result |
| D1 | D | D | D[a] | D[a] | D | D | D | D | D | D | 10 | 0 | D[a] |
| D2 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| D3 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| D4 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| D5 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| D6 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| D7 | D | D | D | D | D | D | D | D | D | D | 10 | 0 | D |
| A1 | A | A | A | A | A | A | A | A | A | A | 0 | 10 | A |
| A2 | D | D | D[a] | D | D | D | D[a] | D[a] | D[a] | D | 10 | 0 | D[a] |
| A3 | D | D | A | A | A | A | D | D | D | D | 6 | 4 | A |
| A4 | D | A | A | A | A | A | A | A | A | A | 1 | 9 | A |
| A5 | A | A | A | A | A | A | A | A | A | A | 0 | 10 | A |
| A6 | A | A | A | A | A | A | A | A | A | A | 0 | 10 | A |
| A7 | A | A | A | A | A | A | A | A | A | A | 0 | 10 | A |
| A8 | A | A | A | A | A | A | A | A | A | A | 0 | 10 | A |
| A9 | A | D | A | D | D | D | A | A | A | A | 4 | 6 | A |
| A10 | A | A | A | A | A | A | D | A | A | A | 1 | 9 | A |
| A11 | A | A | A | A | A | A | D | A | A | A | 1 | 9 | A |
| A12 | A | A | D | A | A | A | A | A | A | A | 1 | 9 | A |
| Total A | 9 | 9 | 10 | 10 | 10 | 10 | 8 | 10 | 10 | 10 | - | - | - |

Results for each FISH probe in each tissue sample classified using the calculated 15% threshold to determine FISH ploidy status for each of the 10 probes. Overall result, ploidy diagnosis assessed by compiled FISH results. D, ICM diploid samples; A, ICM aneuploid samples; Cen, centromeric locus; Tel, telomeric locus. [a]These results were diagnosed as aneuploid rather than diploid or vice versa using the ROC diagnostic threshold of 10%. ICM, image-based cytometry; FISH, fluorescence *in situ* hybridization; ROC, receiver operating characteristic.

two areas, 7 cases with three areas and 1 case with six areas, depending on biopsy size). One additional area was scored for CCND1 only when amplification was found unexpectedly outside the area of high signal counts for other probes. One tissue sample was excluded after the failure of *in situ* hybridization.

In all samples, all connective tissue cell nuclei were diploid, confirming lack of germ line polymorphism at all the loci being assessed in each patient.

*Copy number in ICM diploid samples*. All ICM DNA diploid samples (n=7) had FISH results compatible with diploid status (Table I). Signal counts of three and four, which might represent low copy number gain or cells in S phase, G2 or mitosis, were distributed unequally between samples with counts of three ranging from 0.5 to 14.5% of the total. Only two samples (D6 and D7) had cells with four signals per nucleus, in a total of 1% of nuclei. These data were used to calculate the threshold for aneuploidy.

All probes identified some diploid nuclei with copy number of three and all samples contained some nuclei with copy number three with multiple probes, suggesting variation caused by normal cell cycle. Probes against *PTK2* and *CCND1* produced no nuclei with four signals.

*Copy number in ICM aneuploid samples*. Samples classified as aneuploid by ICM DNA ploidy analysis showed heterogeneous patterns of copy number change with all probes (Table I).

The most frequent changes were copy number changes of three and four copies, amounting to low copy number gain, which were found in between 10 and 30% of cells in all samples. Signal counts of five or higher per nucleus were found in most cases but were detected at only low frequency, in less than 5% of cells. The highest copy number for any locus per nucleus was 12 but this was a rare occurrence (Fig. 1) and cells with only one or two copies of each locus were predominant in most samples. Mean copy number per nucleus ranged from 1.32 to 11.54 and there was general concordance between increased copy number of centromeric and gene-specific telomeric probes (Table SII).

*Gene amplification*. Amplification, FISH signals too numerous and clustered to count (Fig. 2), was a relatively infrequent change observed only with *EGFR* and *CCND1*. *CCND1* amplification was found in four of 12 lesions. One lesion contained a single area that demonstrated amplification of *EGFR* that was sharply demarcated and not noted in adjacent epithelium, was not present in separate tissue slices on the same slide and

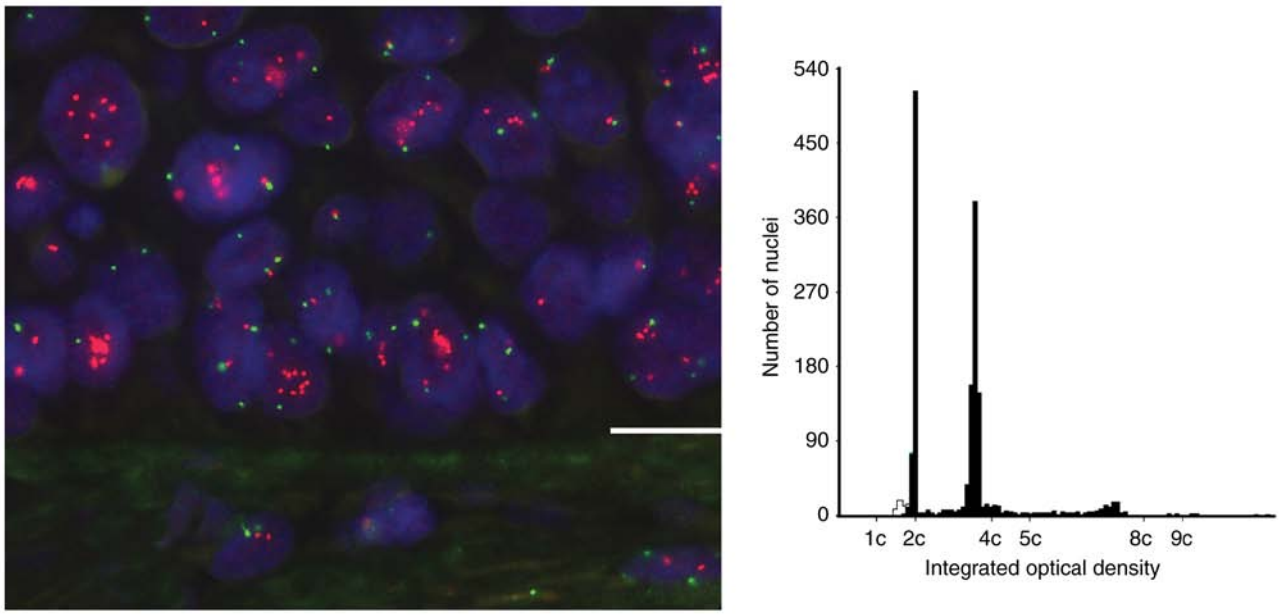


Figure 1. Example showing high copy number gain in an oral dysplastic lesion. Left panel: FISH for *CCND1* (red) and centromeric c11 (green) in the basal and suprabasal cells of the mucosa; basement membrane level at white bar with diploid control connective tissue below and epithelium above. There is a low copy number gain of c11 centromeric locus and high copy number gain of *CCND1*. Right panel: ICM DNA ploidy analysis from adjacent sections revealing a single aneuploid stem line (CV of diploid peak 2.24, 5c exceeding rate 8.8%, aneuploid stem line at diploid index 1.78 (3.6c) with corresponding G2 peak at diploid index 3.8 (7.2c). Original magnification fluorescence *in situ* hybridization, x600. *CCND1*, cyclin D1; ICM, image-based cytometry.

was not in the area showing the most severe dysplasia and not identifiable from changes in routine histological stains (Fig. 2).

*Calculation of FISH threshold to define aneuploidy*. Two methods were used to set a diagnostic threshold value for signal counts that would differentiate diploid status from low copy number gain and both produced similar results.

Using the standard method of Kearney, the threshold was determined over a total of 31,516 diploid cells scored in this study. The highest mean percentage of nuclei with signal counts of three and four plus three standard deviations found with any probe was 12.8% for that against centromere 7 (Table II). Intraobserver error of 2.6% was added and the threshold was set at 15%, rounded to the nearest whole percent. Addition of intraobserver error adds a further element of confidence when defining a cut off level and is an additional precaution that has not been applied in previous studies. This is a conservative threshold; the individually calculated threshold for seven of the probes did not exceed 10%.

ROC analysis calculated from the 47 areas achieved a probability of distinguishing diploid from low copy number aneuploid samples of 0.8 with an area under the curve of 0.951 (95% CI: 0.89-1.00). The minimum distance value to identify the optimum threshold (balancing sensitivity and specificity) was 0.032, providing a sensitivity of 0.833 (95% CI: 0.73-0.94) and specificity of 0.938 (95% CI: 0.87-1.00). Based on ROC, a sample was classified as aneuploid when more than 10% of all nuclei had three or more signals, a slightly less stringent threshold than the Kearney method, but in terms of the result almost directly equivalent for most probes.

*Concordance between FISH and ICM*. Applying the standard calculated threshold, there was complete concordance between FISH and reference standard ICM for diagnosis of the seven diploid samples. For the aneuploid samples, one of 12 ICM samples (A10) was misclassified as diploid, making the results concordant between FISH and ICM in 18 out of 19 samples (94.7%, κ-value 0.89).

From the ROC calculated threshold, one ICM diploid sample (D1) correctly classified as diploid at the 15% threshold was misclassified as aneuploid by FISH. The ICM DNA aneuploid case misclassified as diploid by FISH at the standard calculated threshold was correctly classified as aneuploid.

The 12 ICM DNA aneuploid samples showed a range of copy number aberrations with different probes and most were confirmed to be aneuploid at multiple loci (Tables I and SIII).

*Discordant results*. Misclassification against the ICM reference standard was reviewed (Fig. 3). Sample A10 was clearly aneuploid (CV diploid peak 2.98, aneuploid stem lines at DI 1.88 and 2.2, 5c exceeding rate 3.98%). However, sample D1 lay at the ICM threshold definition of aneuploidy (CV diploid peak 9.9, multiple minor peaks in total comprising 9.98% of the total epithelial nuclei with an average diploid index of 1.95 and a 5c exceeding rate of 0%), just failing to reach the 10% threshold peak size to be diagnosed as aneuploid. This was a true borderline result using both tests.

*Tissue architecture in dysplasia*. The total number of aneuploid cells detected by FISH ranged from 5 to 59% of the basal and suprabasal compartments, but FISH detected fewer aneuploid cells than were found in the aneuploid stem peaks using ICM DNA ploidy in seven cases. These data are shown in Table III together with the values of parameters acquired from ICM DNA ploidy analysis, indicating the number of stem lines detected and their relative DNA content. The results for

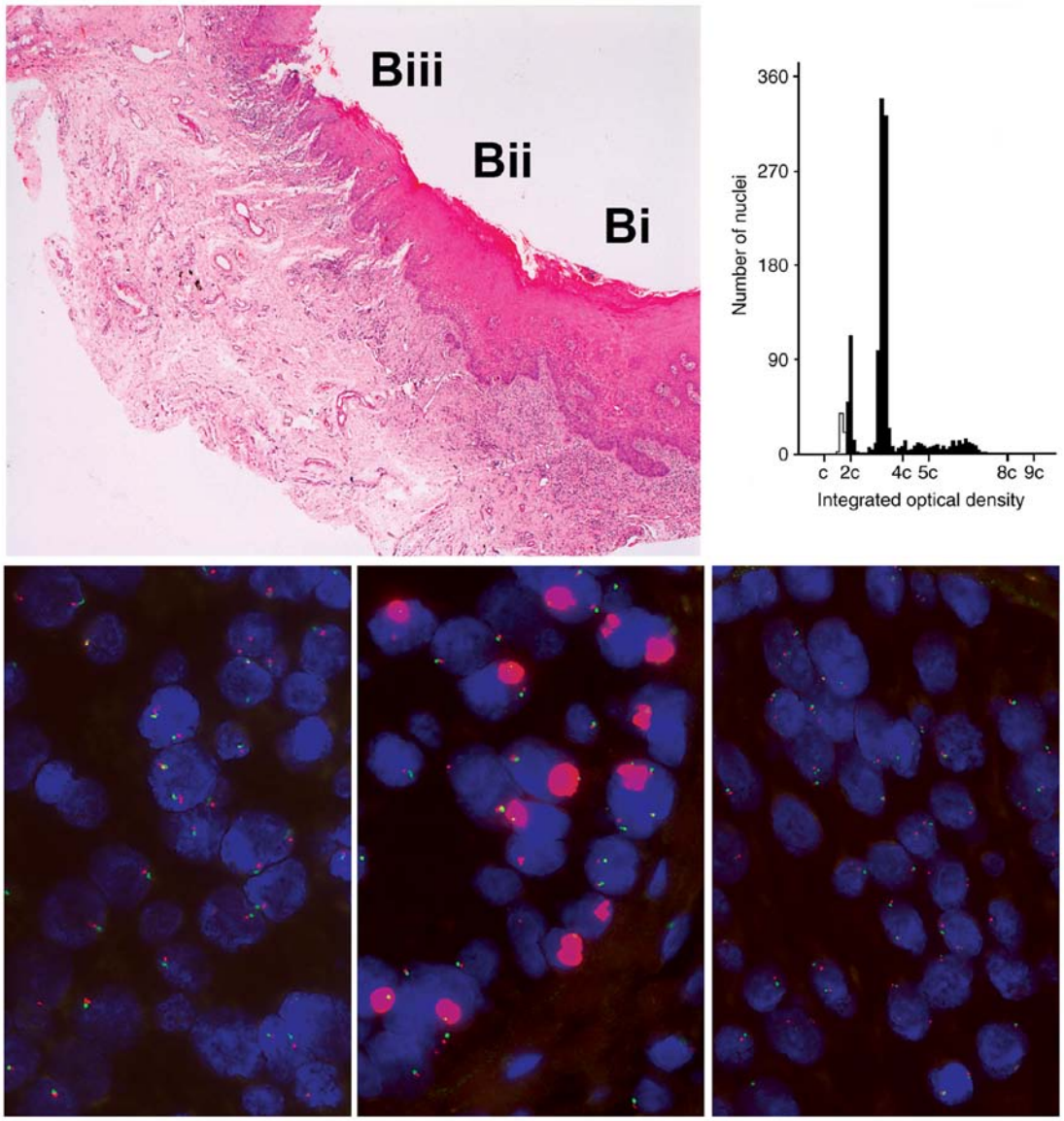


Figure 2. Subclonal structure. Upper left panel: hematoxylin and eosin (H&E)-stained section. One of 3 adjacent tissue slices from sample A20; a white lesion from the lateral tongue in a female non-smoker aged 30 years who developed severe dysplasia at this site 2 years after this biopsy and then squamous cell carcinoma at the same site 4 years after this biopsy. Upper right panel: Image-based DNA ploidy analysis histogram reveals an aneuploid stem line population (epithelial nuclei black bar, control fibroblasts and lymphocytes white bar, CV of diploid peak 2.89, 5c exceeding rate 10.14%, aneuploid stem line at diploid index 1.64). Lower panel: FISH for *EGFR* (red) and centromeric c7 (green). The areas labelled Bi, Bii and Biii (upper left panel; annotations match supplementary data online) were assessed separately; right, area Bi and left, area Biii are diploid at the *EGFR* locus but there is marked *EGFR* amplification in the central zone Bii. This area is not detectable in the routine levels on each side of the section assessed and none of the three areas shown showed dysplasia. Original magnification fluorescence *in situ* hybridization, x600. *EGFR*, epidermal growth factor receptor.

each probe were constant between areas in the same sample. Gene amplification was not found within zones of copy number gain and these appeared independent processes in the samples tested.

## Discussion

This study utilized two different methods to assess chromosomal instability through copy number gain in oral epithelial dysplasia. Image-based cytometry (ICM) DNA ploidy analysis is a gross DNA measurement technique that works well to predict development of carcinoma in clinical practice (12,14,23,24). It can identify stable stem lines or clones within the lesion but reveals nothing of the tissue architecture. In contrast, *in situ* hybridization detects specific sequence changes in DNA in single cells and can detect the spatial arrangement of DNA changes at a tissue organizational level. However, fluorescence *in situ* hybridization (FISH) is inefficient to detect deletions as some of the nuclei will always be sectioned and incomplete, mimicking deletion and the small numerical reductions make it difficult to detect statistically significant reductions.

The FISH target sequences used here were selected as markers of sites of common amplification in oral potentially malignant disorders (OPMD), based on our previous research (25) and published data (18, 26-29). The specific sequences at 3q28, 7p11.2, 8q24.3, 11q13.3 and 20q13.12 were in genes *TP63, EGFR, PTK2, CCND1* and *MMP9*. However, these genes were chosen for their good characterization

Table II. Total mean percentage of nuclei plus three standard deviations of copy number 3, 4, and 3 and 4 for each probe target.

| | Mean percentage of nuclei + 3SD | | |
|---|---|---|---|
| Locus | Copy number 3 | Copy number 4 | Copy number 3 and 4 |
| Cen 3 | 3.88 | 0.99 | 4.87 |
| 3q28 | 1.97 | 0.57 | 2.54 |
| Cen 7 | 12.18 | 0.58 | 12.76 |
| 7p11.2 | 10.06 | 0.41 | 10.47 |
| Cen 8 | 4.60 | 0.41 | 5.01 |
| 8q24.3 | 4.84 | 0.00 | 4.84 |
| Cen 11 | 6.86 | 0.41 | 7.27 |
| 11q13.3 | 2.26 | 0.00 | 2.26 |
| Tel 20p | 8.86 | 0.91 | 9.77 |
| 20q13.12 | 3.54 | 0.57 | 4.12 |

3SD, three-times the standard deviation.

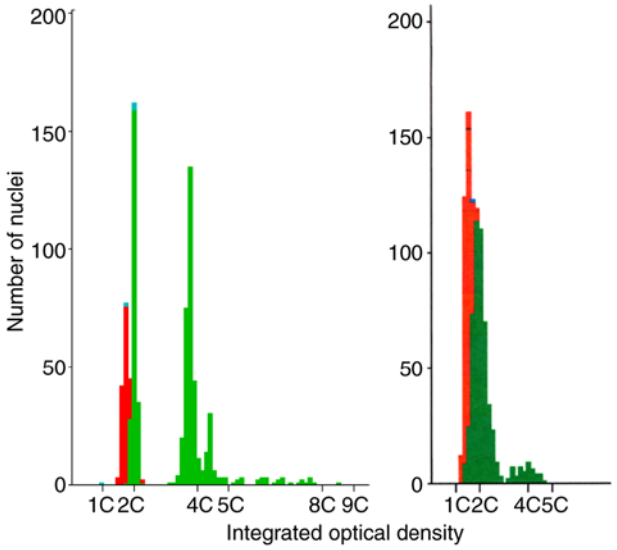


Figure 3. ICM DNA ploidy histograms for FISH misclassified samples. Sample A10 left, sample D1 right. ICM, image-based cytometry; FISH, fluorescence *in situ* hybridization.

and location in commonly amplified regions, rather than any putative role in malignant transformation. We attach no significance to these specific genes in interpreting our data. *EGFR* and *CCND1* have been used in previous similar studies but the probes for 3q, 8q and 20q are novel. Centromeric, and one remote telomeric probes were included to assess possible chromosomal duplication, two of which were novel.

FISH signal counting was performed in continuous runs of basal and transit amplifying cells in sections optimized to minimize nuclear loss but reduce nuclear overlap. Standard clinical diagnostic counting and diagnostic techniques were used, but we counted many more cells than are normally counted in similar studies (20,29), and sampled across a much wider spatial range in each sample. Control cells were reproducibly diploid with all probes, excluding false-positive results from pseudogenes. ICM DNA samples were taken from adjacent sections, with microdissection if required, to ensure both samples were as similar as possible.

There are no standardized criteria to define aneuploidy by FISH. Signal counts of three or four per nucleus could represent either cells in S, G2 phase or mitosis, or low-level copy number gain. In other similar studies, counts of three or four per nucleus have been interpreted as either low copy number gain (30), trisomy and tetrasomy (20), or trisomy or polysomy (29), sometimes without statistical support. We applied the standard thresholding method (22), which is well established (31-34) to define a diagnostic threshold and our data supports Poh *et al* (30) in defining cases with copy number variation of three and four as having low copy number gain.

Using the maximum threshold value of 15% to exclude false-positive results, only one incorrect classification of diploid status was made by FISH, compared to reference standard ICM. Using the diagnostic criteria applied by Poh *et al* (30), this sample (A10) would have been classified as low copy number gain.

Receiver operating characteristic (ROC) analysis, although a more accurate method, could achieve only a low statistical power in the sample size, despite a high area under the ROC curve. Applying the calculated threshold classified all ICM DNA aneuploid samples correctly but generated one false-positive aneuploid result. A threshold of 10% was used by Poh *et al* without calculation, adopted from earlier studies in lung carcinoma (30). There are no published data on threshold calculations in other similar publications (20,29). With both the ROC analysis and the standard method, the FISH assay has good concordance with ICM DNA ploidy, good sensitivity and specificity.

Recently, Siebers *et al* showed an association between malignant transformation and aneuploidy assessed by two centromeric probes, for chromosomes 1 and 7 (20). Using only two FISH markers the agreement with ICM ploidy status was only 63% compared to 90% agreement with the present 10 marker panel. Addition of more target loci would be expected to increase the predictive value of the panel, and its agreement with ICM, but with diminishing returns as additional probes are selected against less frequently amplified loci. Our panel of 10 probes almost reached equivalence to DNA ICM, and thus exceeded the predictive value of conventional dysplasia grading (35).

The clonal structure of precancer is well defined in the colon (36) and at other sites. Dysplasia of the oral mucosa is often sharply demarcated laterally and this is generally considered to indicate a clonal structure, as has been identified by X-linked histochemical methods (37) and, less specifically, by differences in loss of heterozygosity patterns within lesions (38). Oral mucosa has a similar stem cell and clonal structure to skin (39), which is better developed in epithelia with well-developed rete processes (40). Understanding the clonal structure of premalignant oral epithelium is important as it may affect therapeutic interventions for field change and reflect the initial clonality of oral squamous carcinomas. Recent molecular data suggest that oral dysplasia is clonal and has neutral clonal evolution rather than being epithelium that is progressively being completely replaced by clones selected by greater growth potential (41). This

Table III. Comparison between ICM DNA ploidy parameters and FISH aneuploid cell detection.

| Sample number | Diploid index of stem peak(s) on ICM | % epithelial cells in stem peak on ICM | % 5c exceeding frequency on ICM | Total % aneuploid epithelial cells on ICM | Total % aneuploid cells on FISH (all probes) | Number of FISH probes showing aneuploidy |
|---|---|---|---|---|---|---|
| A1 | 1.9<br>2.2 | 13<br>3[a] | 1.00 | 17 | 33.7 | 10 |
| A2 | 1.88<br>2.2 | 47<br>8[a] | 4.0 | 59 | 5.0 | 0[b] |
| A3 | 1.1 | 29 | 0 | 29 | 6.7 | 4 |
| A4 | 1.6 | 10 | 0.4 | 10.4 | 12.0 | 9 |
| A5 | 1.8 | 42 | 3.1 | 45.1 | 27.4 | 10 |
| A6 | 2.0 | 22 | 1.8 | 23.8 | 29.7 | 10 |
| A7 | 3.5 | 87 | 11.1 | 87 | 47.4 | 10 |
| A8 | 2.8 | 11 | 11.5 | 22.5 | 59.3 | 10 |
| A9 | 1.8 | 44 | 8.9 | 52.9 | 10.7 | 6 |
| A10 | 1.6 | 15 | 0.8 | 15.8 | 24.2 | 9 |
| A11 | 2.18 | 51 | 11 | 62 | 19.6 | 9 |
| A12 | 1.6 | 66 | 10.1 | 76.1 | 37.7 | 9 |

Comparison between ICM DNA ploidy parameters and FISH aneuploid cell detection, using the calculated aneuploid threshold of a maximum of 15% nuclei with copy number 3, 4 or higher. [a]These smaller peaks do not reach the diagnostic threshold of 10% for aneuploidy and DNA ICM ploidy status is defined by the larger peak. [b]This sample was classified as aneuploid by FISH using the ROC calculated threshold of 10%. ICM, image-based cytometry; FISH, fluorescence *in situ* hybridization; ROC, receiver operating characteristic.

is consistent with research suggesting that cancer pathways in dysplasia are frequently inhibited rather than activated (21). This FISH analysis has revealed insights into the spatial molecular architecture of oral dysplastic lesions that are not observed by other methods and are consistent with the concept that some aneuploid cells in dysplasia are incapable of maintaining a pure clonal population.

Our results showed that amplification at a specific locus is only carried by a minority of epithelial cells in any lesion and that clearly aneuploid samples rarely contained cells with high copy number gains, consistent with ICM results that OPMD usually only harbor stem lines with DNA content of 3c or less. FISH showed that most aneuploid lesions had amplification with the majority, or all, probes, suggesting one abnormal clonal population.

DNA ICM is the more sensitive technique, detecting many more aneuploid cells than FISH in most samples, and revealing a single stem line population in 10 of 12 aneuploid samples. Our results show that ICM DNA aneuploid stem lines are relatively homogeneous at a molecular level, at least within the detection limits of both techniques and 10 probes. Two aneuploid samples lacked ICM stem line peaks, and one was aneuploid with all 10 FISH probes despite apparently being ICM non-clonal.

Concordance between centromeric and telomeric amplification suggests that whole chromosomes or large parts of chromosomes are duplicated, consistent with the concept that the cause of DNA ploidy anomalies is usually non-disjunction.

Multiple copy gene amplification involved only *CCND1* (four samples) and *EGFR* (one sample) and both loci showed distinctive patches of clustered signals with sharp boundaries suggesting a clonal architecture. *CCND1* amplification co-localized with severe dysplasia, but *EGFR* amplification did not correlate with dysplasia in routine sections and the affected epithelium appeared normal. Both of the other adjacent tissue slices in the block from this latter lesion were diploid throughout, showing the size of the clone carrying *EGFR* amplification to be small, less than 2 mm across.

*EGFR* and *CCND1* amplifications have been previously investigated in 35 oral dysplastic lesions and high copy number was strongly associated with malignant transformation (30). That study also showed amplification of *EGFR* and *CCND1*, but in fewer cases than in the present study. In another study of 20 oral premalignant lesions, only one had gene amplification (29), together suggesting it is a relatively infrequent change.

In summary, a panel of 10 FISH probes against loci that frequently show increased copy number in oral dysplasia revealed that aneuploid cells in our oral dysplasia samples were interspersed with normal diploid cells so that dysplastic epithelium did not comprise a uniform clonal population of cells. In all cases, aneuploid cells formed only a minority population and high copy number gain was unusual. These findings add weight to the suggestion that oral dysplasia contains cells with inhibited cancer pathways (21) and develops through neutral clonal evolution (41) rather than being a progressive replacement of epithelium by clones with a growth advantage on a relentless pathway to carcinoma. In any one lesion the aneuploid cells had amplification at all or almost all loci tested, matched to chromosome-specific loci and therefore the result of chromosome or large fragment duplication, making

non-disjunction the likely cause. A few small clones were detected by gene amplification. The present study utilized the broadest panel of FISH probes applied to oral dysplasia and the panel had almost equal ability to detect aneuploidy as image-based DNA ploidy analysis.

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**Availability of data and materials**

The datasets used and analysed in the present study are available from the corresponding authors on reasonable request.

**Authors' contributions**

ZMZ conceived and performed the experiments, analyzed the data, carried out the statistical analysis and wrote the manuscript. EWO conceived and designed the study, carried out acquisition of the samples and edited the manuscript. AS contributed to the study design and selection of markers. MN and MT provided expert advice on experimental protocol and facilitated the interpretation of the results. All authors reviewed, edited and approved the final version of the manuscript, and all agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

This study complies with UK guidelines and legislation and use of material and individuals' information has been specifically approved by the UK Patient Information Advisory Group [PIAG reference 4-09(f)2003].

**Patient consent for publication**

Specifically exempted under the ethics approval above.

**Competing interests**

The authors declare that they have no competing interests.

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