Comparative evaluation of genetic assays to identify oral pre-cancerous fields

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BACKGROUND: Oral squamous cell carcinomas often develop in a pre-cancerous field, defined as mucosal epithelium with cancer-related genetic alterations, and which may appear as a clinically visible lesion. The test characteristics of three genetic assays that were developed to detect pre-cancerous fields were investigated and compared to histology.

METHODS: In total, 10 pre-cancerous fields that were not visible at clinical inspection and gave rise to malignant transformation based on an identical TP53 mutation in tumor and mucosal epithelium in the surgical margin, as well as 10 normal oral mucosa specimens were analyzed for numerical chromosomal changes with multiplex ligation-dependent probe amplification (MLPA), for loss of heterozygosity (LOH), with microsatellite PCR and for DNA index alterations with DNA image analysis.

RESULTS: No alterations were detected in normal tissue by either of the assays. Both MLPA and LOH assays detected all pre-cancerous fields. DNA cytometry identified aneuploidy in four of 10 pre-cancerous fields, while the corresponding tumors that developed in these fields were shown to be aneuploid.

CONCLUSIONS: Both the MLPA and LOH assay seem suitable for screening pre-cancerous fields in subjects at high risk for oral cancer even in the absence of clinically abnormal appearing oral mucosa. Measurements of DNA index might be valuable to determine the time to progression.


Keywords: DNA ploidy; field cancerization; leukoplakia; loss of heterozygosity; MLPA

Introduction

Oral cancer is a significant public health threat accounting for 270,000 new cases annually worldwide (1). Approximately, 80% of all oral cancers are oral squamous cell carcinomas (OSCC) that arise in the mucosal linings. Despite advances in therapy leading to better local control, the long-term survival rates of patients with OSCC have only marginally improved during the last 20 years (2). The prognosis of OSCC is strongly related to the stage: small tumors that have not yet spread to the regional lymph nodes of the neck have a much more favorable prognosis than more advanced tumors that already have metastasized via the lymphatic compartment (3).

On the basis of the large difference in prognosis between early and advanced stage tumors, one would envision that screening programs for oral cancer might reduce cancer-related deaths. A limited number of studies have been carried out during the last decade and thus far this approach has yielded no (4–6) or limited (7) success. An important reason might be that the tumors progress rapidly and consequently the screening interval needs to be short.

A potentially more successful strategy might be the identification of pre-cancerous fields in the mucosal epithelium that precede the carcinomas. It has been well established that OSCC is the result of a multistep process that progresses over several years (8). In 2003, Braakhuis (9) proposed an adapted progression model of oral cancer in which they described that OSCC develops within a mucosal field with cancer-associated genetic alterations that replaced part of the normal epithelium in the oral cavity. Recently, these field alterations in tumor margins of oral cancer patients could be visualized by autofluorescence (10). The clinical relevance of the presence of these fields is well established as it was shown that these cause local recurrences and second primary tumors in treated oral cancer patients (11–13).
The adapted progression model was in part based on the genetic observations in the mucosa surrounding the tumors but in part also based on existing clinical experience as it is known that a subgroup of pre-cancerous fields can be identified clinically as white or red lesions in the oral mucosa, designated as leukoplakia or erythroplakia, respectively. The clinical management of these visible oral lesions routinely involves biopsy for histological examination and grading for dysplasia. The presence and grade of dysplasia will influence treatment, usually excision and follow-up. However, as only a minor subgroup is macroscopically visible, the use of clinical examination to identify oral pre-cancerous fields as a method of screening thus meets limitations (9, 14, 15).

The knowledge that a large proportion of OSCC developed in these pre-cancerous fields characterized by cancer-associated genetic changes now opens the possibility to screen for the presence of such pre-neoplastic changes in the absence of clinically visible lesions (14). Taking random biopsies for histological examination is not an attractive option for routine screening. Standard cytology, a routine method used for cervical cancer and pre-cancer screening, has been reported to be not successful for use in the oral cavity (16). Recent studies have shown that cancer and pre-cancerous fields might be detected by non-invasive methods employing microsatellite analysis to determine tumor-specific allelic imbalance in exfoliated oral mucosal cell samples from patients with OSCC (17). In addition, multiplex ligation-dependent probe amplification (MLPA) has been shown to be a promising method for non-invasive screening of high-risk populations to detect oral pre-cancerous fields (18). The MLPA assay allows the measurement of numerical chromosomal alterations, gains and losses, at up to 40 target locations in a single PCR run. Probes that differ in length are hybridized against a specific location in the genomic DNA, ligated, PCR amplified by a general primer pair, and analyzed by capillary electrophoresis. This set up allows a quantitative analysis of the copy numbers of multiple chromosomes. Apart from these genetic markers, also the ploidy status has been used to examine cytologic scrapings of visible oral lesions, and this approach might be suited as well to investigate clinically normal appearing oral mucosa (19, 20). Measurement of allelic imbalance, commonly known as loss of heterozygosity (LOH), MLPA, and DNA ploidy analysis are three potential screening methods to detect pre-cancerous fields that could be employed on samples obtained by non-invasive methods. Our study compares the potential of these methods for the detection of genetically altered fields in clinically normal appearing tissue. The results are important for the future development of a non-invasive screening assay with scraped cells as sample source.

**Materials and methods**

*Patients and tissue samples*

From previous studies, we selected primary oral tumor samples with corresponding resection margins, obtained between 1993 and 2000, that were shown to contain a pre-cancerous field clonally related to the tumor by LOH analysis. All specimens were fixed by formalin and subsequently embedded in paraffin. Specifically, these samples were selected from a large series of 40 tumors and about 150–200 surgical margins previously studied, as the tumors and the mucosal epithelium of the corresponding surgical margins had the same *TP53* mutation (12–14, 21). This was considered strong evidence that tumor and pre-cancerous field in the margin were clonally related, and that the tumor arose in this field. Six primary oral tumor samples with corresponding margins and two metachronous multiple primary tumors of two patients with corresponding resection margins were selected for this investigation, analyzing in total 10 tumors with 10 corresponding margins. All tissues were available in paraffin blocks. All hematoxylin and eosin (HE)-stained slides were examined independently by three experienced pathologists for dysplasia according to the standard criteria of the World Health Organization. A consensus score was defined after a review session (22). Tissue characteristics are summarized in Table 1.

Normal oral mucosa was derived from 10 paraffin-embedded surgical specimens of uvulas from patients who underwent surgical treatment for snoring. Blood and exfoliated cells of the oral mucosa obtained from a number of other non-cancer subjects served as control samples. The studies were approved by the Institutional Review Board, and in accordance with Dutch medical ethical guidelines. Patients in prognostic studies were enrolled after written informed consent.

For serial dilution experiments, four cell lines were used: human HNSCC cell line UM-SCC-22A obtained from Dr T.E. Carey (Ann Arbor, MI, USA); FaDu, an established human hypopharyngeal squamous cell carcinoma line provided by the American Type Culture Collection (Rockville, MD, USA); VU-SCC9917T, a cell line established at our own Department, and primary cultured oral keratinocytes that were immortalized by oncogenic (large T-antigen) transformation (S.J. Smeets, unpublished data). Cells were routinely cultured in Dulbecco’s modified Eagle’s Medium with 5% fetal calf serum or keratinocyte growth medium.

**Table 1** Tissue characteristics

<table>
<thead>
<tr>
<th>Patient tumor</th>
<th>Site</th>
<th>Dysplasia resection margin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tongue border R</td>
<td>Mild</td>
</tr>
<tr>
<td>2-a</td>
<td>Buccal mucosa R</td>
<td>Mild</td>
</tr>
<tr>
<td>2-b</td>
<td>Palate</td>
<td>Moderate–severe</td>
</tr>
<tr>
<td>3-a</td>
<td>Alveolar process R</td>
<td>Mild</td>
</tr>
<tr>
<td>3-b</td>
<td>Buccal fold superior R</td>
<td>Mild</td>
</tr>
<tr>
<td>4</td>
<td>Alveolar process</td>
<td>Severe</td>
</tr>
<tr>
<td>5</td>
<td>Floor of mouth R</td>
<td>Severe</td>
</tr>
<tr>
<td>6</td>
<td>Tongue border R</td>
<td>Moderate–severe</td>
</tr>
<tr>
<td>7</td>
<td>Tonsil R</td>
<td>Severe</td>
</tr>
<tr>
<td>8</td>
<td>Supraglottic</td>
<td>Severe</td>
</tr>
</tbody>
</table>

R, right.
Dissection
For LOH and MLPA analysis, tumor, and pre-neoplastic mucosal epithelium from the resection margins were enriched by microdissection. From these specimens, 10-μm paraffin sections were cut and mounted on microscopic glass slides. The first and last tissue sections were stained with HE for histological analysis and to guide dissection. The other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue in phosphate-buffered saline (PBS) and manually dissected under a stereomicroscope.

Isolation of DNA
The microdissected tissues as well as the nucleated cells of blood samples obtained by hypotonic lysis were treated with 1 mg/ml of proteinase K for 24 h at 52°C in 100–300 μl buffer containing 100 mM Tris–HCl (pH 9.0), 10 mM NaCl, 1% sodium dodecyl sulfate, and 5 mM ethylene-diaminetetraacetate (EDTA) (pH 8.2). The DNA was purified by phenol–chloroform extraction and collected by ethanol precipitation using 2 μl of glycerol as carrier. The DNA was dissolved in 20 μl LoTE buffer (3 mM Tris–HCl, 0.2 mM EDTA, pH 7.5).

LOH analysis
Allelic loss was assessed on the enriched (pre-)neoplastic cells using 12 microsatellite markers located at chromosomes 3p, 9p, 11q, and 17p. The corresponding cancer genes at these chromosomal locations are: at 3p gene formerly known, but possibly the FHIT gene, at 9p the p16 gene, at 11q the cyclin D1 gene, and at 17p the TP53 gene. This marker panel was selected based on the following criteria: high percentage of LOH in HNSCC and pre-neoplastic lesions (9, 14), a high percentage of informativity, and amplimer lengths that can be combined for multiplex sequence runs (23). The following markers were used: D3S1766, D3S1029, D3S1293, D9S171, D9S162, D9S157, D11S1883, D11S1369, D11S2002, CHRN1, TP53, and D17S1866. Primer sequences were obtained from the genomic database UniSTS. LOH analysis was performed as described previously, including stutter correction (12, 14, 24).

We analyzed various tissue specimens in this study including archival specimen. To circumvent differences in genetic data on the basis of the type of material, we used very strict methods and criteria. We always include a normal reference DNA from the same paraffin block for LOH analysis and standardize the amount of input DNA in the assays.

Previously, we scored LOH when one allele was decreased by more than 50% in the sample when compared with the same allele in normal control DNA, an internationally accepted but arbitrary standard (23) that allows some variation in the allelic ratios and the presence of normal cells in the sample. However, the analytical variation for the 12 selected LOH markers might be larger than these cut-off points, and we therefore calculated for each marker the cut-off points limiting the 99% confidence interval (CI) of the mean value by performing 20 repeated analyses on a control blood DNA sample informative for all markers. The ratios limiting the 99% CI corresponding to non-altered DNA of each marker is presented in Table 2. CHRN1 was the least performing marker with 99% CI cut-off values to call LOH determined as less than 0.7 and greater than 1.42, but still well within the ratios of 0.5 and 2.0 used.

MLPA analysis
Multiplex ligation-dependent probe amplification analysis, using the OSCC probe set (MRC-Holland, Amsterdam, The Netherlands) was performed on the microdissected samples as described earlier (18, 25). For each MLPA probe in the OSCC probe set, we determined specific cut-off values for gain or loss, using the values limiting the 99.99% CI as determined on 20 blood samples of 20 non-cancer subjects. When the upper limit of the CI was exceeded, a numerical gain was scored while values below the lower limit defined a numerical loss (18).

DNA ploidy analysis
All tissues were analyzed with DNA image cytometry (ICM). For ICM, two or three 50-μm sections were cut from paraffin-embedded tissue specimens and cell suspensions were prepared according to the Hedley procedure (26). To increase the sensitivity of the analysis, the dysplastic areas were selected and when possible, the parts of the tissue peripheral to the lesions were removed. Cytopsins were prepared (15 min at 3000 rpm) and stained by the Feulgen procedure according to the consensus of the European Society for Analytical Cellular Pathology (27). In short, cytopsins were placed in 5 N HCl for 30 min at 27°C, rinsed in distilled water for 5 min, stained with fresh Schiff’s reagent for 45 min, and washed in running tap water for 15 min.
DNA content of the stained nuclei was measured and analyzed by ICM according to an established protocol (28). In short, monolayers were analyzed with the use of an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with a 20× plan apochromat (numerical aperture of 0.6) and a projective magnification of 1.6×. Images were recorded by a XC-77-CE CCD black and white camera (Sony, Köln, Germany). The slide was moved with an automatic scanning stage (Märzhäuser, Wetzlar, Germany) with a step size of 0.25 μm. The specimen was illuminated with a stabilized halogen light source and filtered with a green monochrome filter (λ = 550 nm, Δλ = 10 nm). Images of 512×512 picture elements were digitized with a Matrox Meteor II PCI frame grabber (Matrox Electronic Systems Ltd. Imaging, Dorval, Canada) in 8-bit gray value resolution. The pixel-to-pixel distance at the specimen level was 0.33 μm. Image processing was performed on a dedicated personal computer with a color monitor at a spatial resolution of 1152×900 in 24-bit. Prior to every image analysis session, Köhler illumination was applied. The nuclei of at least 1000 cells were measured, and lymphocytes and fibroblasts were included as internal controls.

For DNA flow cytometry (FCM), cell lines resuspended in RPMI cell culture medium and 5% fetal bovine serum were pelleted (5 min at 500 rpm), washed with PBS, and subsequently stained with 4′, 6-diamidino-2-phenyl-indole (DAPI). FCM was performed within 3 h after DAPI staining with a Partec PAS II mercury lamp based flow cytometer (Partec Instruments, Muenster, Germany) using trout erythrocytes as external control cells.

**Criteria for the classification of DNA content**

Image cytometry and FCM-DNA histograms were analyzed with the MultiCycle AV computer program (Phoenix Flow Systems, San Diego, CA, USA) according to a previously developed protocol (29). Based on the DNA index, ICM cases were classified into three subclasses as follows: DNA diploid (only one peak during G0 or G1 phase), 4c nuclei during peak G2 phase <10% of total, or 5c nuclei <0.1% of total), tetraploid (4c peak during G0 or G1 phase together with 8c peak during G2 phase or 4c nuclei during peak G2 phase >10% of total), and aneuploid [presence of aneuploid peaks (3c, 5c, 7c, 9c) or number of nuclei more than 5c or 9c >0.1% of total] (25). FCM cases were classified in different ploidy classes as published by Uyterlinde et al. (30).

**Non-invasive sampling**

Exfoliated cells were scraped from the oral mucosa of non-cancer controls using a small disposable brush (Omnident®, Dental Union, Nieuwegein, The Netherlands) and prepared as described previously (18).

**Serial dilutions**

For analysis of the analytical sensitivity of MLPA and LOH analysis, we mixed DNA isolated from peripheral blood from non-cancer controls with DNA isolated from OSCC cell line VU-SCC-9917T. We used the following five dilutions (n = normal DNA, T = tumor DNA): 100% n, 75% n, 25% T, 50% n, 50% T, 25% n, 75% T, and 100% T.

To assess the analytical sensitivity of DNA ploidy analysis by FCM, we selected three different cell lines: UM-SCC-22A (diploid), FaDu (aneuploid), and SV40 transformed oral keratinocytes (tetraploid). The aneuploid cell line FaDu and the tetraploid transformed oral keratinocytes were mixed with the diploid cell line UM-SCC-22A in serial dilutions of 1/10000, 1/1000, 1/100, 1/10 and 1/4, 1/2, respectively.

**Results**

To assess the analytical sensitivity of the various screening methods, we analyzed 10 pre-cancerous fields in the resection margins of excised tumors by the MLPA, LOH, and DNA ploidy assays (31). The presence of the fields and the clonal relation to the tumor was based on an identical TP53 mutation, strongly indicating that the tumors originated in these pre-cancerous fields. The results of all assays are shown in Fig. 1. The MLPA assay and LOH assay demonstrate genetic alterations in all 10 pre-cancerous fields. In contrast, only four out of 10 fields were detectable by a change in DNA index, in all cases determined as aneuploidy. In these four aneuploid fields also so called stem lines were found, indicated by 5c peaks. In addition, we analyzed the DNA index of all corresponding tumor samples. Only six of 10 tumors could be evaluated and were found to be all aneuploid. Although the limited sample size needs to be taken in consideration, we found that in three cases both the tumor and the surgical margin were aneuploid. In three other cases, however, the surgical margin was found to be diploid while the tumor was aneuploid (Table 3).

To determine the detection limits of all assays, we performed analysis of serial dilutions of normal and aberrant samples. In the MLPA assay, the genetic alterations were just detected when 50% or more tumor DNA was mixed with normal DNA. In the LOH assay, we found the genetic alterations when 75% or more tumor DNA was mixed with normal DNA. The aneuploid cell line could be detected in a background of diploid cells until dilution 1/10, meaning that the detection of only 10% of aneuploid cells in a sample is feasible. The tetraploid cell line could be detected until dilution 1/4, meaning that the presence of more than 25% of tetraploid cells in a sample is detectable (Fig. 2).

All three assays were also performed on 10 mucosal tissue samples of non-cancer controls to determine the analytical specificity. With MLPA and LOH, all samples were free of genetic alterations. All samples were also found to be diploid. MLPA and LOH assays were performed in addition on DNA derived from exfoliated cell samples taken in duplicate from 20 young (<30 years) non-smoking, non-cancer controls and no genetic alterations were found.

Finally, we correlated the findings obtained with the various assays, including dysplasia grading. When the
Figure 1  Multiplex ligation-dependent probe amplification (MLPA), loss of heterozygosity (LOH), DNA ploidy assays, and dysplasia score were compared to identify 10 oral precursor fields that were macroscopically not visible as clinically identifiable lesions. The fields surrounded a primary OSCC and showed the same TP53 mutation as the primary tumor, indicating a clonal relationship and suggesting that the tumor arose in this field. For the MLPA, numerical losses (light gray) and gains (dark gray) are indicated. For the LOH, allelic loss is indicated in gray (more than 50% change in allele ratio as compared to normal DNA from the stroma). Particularly, the larger microsatellite markers are very difficult to analyze on formalin-fixed paraffin-embedded samples. OSCC, oral squamous cell carcinomas; NI, not informative; NA, not analyzable.
LOH and MLPA results for the separate chromosome arms 3p, 9p, 11q, and 17p were compared, a highly significant difference ($P < 0.001$ by Fisher’s exact test) was found for the markers at 17p. With MLPA, only three of 40 17p markers showed genetic changes, while with LOH this was 12 of 25 informative markers, confirming that with LOH other but very relevant genetic changes are detected. When we analyzed the association of genetic changes with DNA index status, it was found that the number of genetic alterations measured by either the MLPA or the LOH assays in diploid margins did not show significant differences with the number of genetic alterations in the aneuploid margins ($P$-values 0.92 and 0.31 by Fisher’s exact test, respectively). Intriguingly, there were differences related to dysplasia grading. The MLPA results showed a significantly increased number of numerical genetic alterations in moderate-severe dysplasias when compared with mild dysplasias ($P$-value of 0.008 by Fisher’s exact test). This difference was not seen in the relative number of allelic losses ($P$ value = 1). Remarkably, the presence of aneuploidy also seemed not related to dysplasia grading.

Discussion

Screening for pre-cancerous changes is evaluated or even already part of population-based screening programs for several tumor types. The most well-known example of a screening program focusing not only on cancer detection but also on detection of pre-cancerous changes is that for cervical cancer. The incidence and mortality of invasive cervical carcinoma have decreased over the last 30 years in the developed world, a fact which has been attributed mainly to the implementation of cytological screening programs (Papanicolaou test). Studies that have evaluated cervix cytology screening show an estimated sensitivity of 22–29% and a specificity ranging from 85 to 100% (32).

Screening for oral pre-cancer in the general population at high age might not be cost-effective. We foresee that application of non-invasive screening will be applied in high-risk groups such as leukoplakia patients and treated oral cancer patients. Also other populations at high risk for oral cancer either by genetic predisposition or a specific lifestyle might qualify for screening programs (33).

Our study was performed to evaluate test characteristics of three different genetic assays that might be suitable to screen for oral pre-cancerous fields in apparently normal looking mucosa. The size of the samples is not very large, but very unique and selected from a series of 40 tumors and about 150–200 surgical margins. Both tumors and precursor lesions were sequenced for $TP53$ mutations and for these sample sets, enough material was present for these comparative analyses. These particular tumors apparently arose from the non-visible field surrounding them based on the identical $TP53$ mutation. A specific OSCC probe set was used for MLPA analysis and the cut-off values were based on a previous study (18). Based on the present results, the MLPA assay seems a suitable assay to screen for oral pre-cancerous fields. The MLPA assay was able to detect alterations in all pre-neoplastic fields and showed no genetic alterations in normal oral mucosa and exfoliated cell samples from non-cancer controls. However, limitations of the MLPA assay are that automatization might be difficult and that the assay is very sensitive to DNA input (data not shown), which decreases the robustness in heterogeneous sample sets.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>DNA ploidy</th>
<th>DNA ploidy</th>
<th>surgical margin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NE</td>
<td>Diploid</td>
<td></td>
</tr>
<tr>
<td>2-a</td>
<td>Aneuploid</td>
<td>Aneuploid</td>
<td></td>
</tr>
<tr>
<td>2-b</td>
<td>Aneuploid</td>
<td>Diploid</td>
<td></td>
</tr>
<tr>
<td>3-a</td>
<td>Aneuploid</td>
<td>Aneuploid</td>
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<tr>
<td>3-b</td>
<td>Aneuploid</td>
<td>Aneuploid</td>
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<tr>
<td>4</td>
<td>NE</td>
<td>Diploid</td>
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<td>5</td>
<td>NE</td>
<td>Aneuploid</td>
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<td>7</td>
<td>Aneuploid</td>
<td>Diploid</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>NE</td>
<td>Diploid</td>
<td></td>
</tr>
</tbody>
</table>

NE: not evaluable by lack of material.
An intriguing observation is the large difference between numerical chromosomal changes at 17p and allelic losses. Numerical changes were hardly found while allelic losses were found in almost half of the cases at the same chromosomal region. This strongly indicates that copy neutral events occurred at 17p, the target locus of TP53, and supports previous findings that copy number changes are less frequent than allelic losses (34, 35). Obviously, the data should be interpreted with caution as the exact locations and number of markers tested differ between the MLPA and LOH assays. Nonetheless, the difference is remarkable and points to a peculiar role of the TP53 gene in oral cancer progression.

Another interesting observation is the association of genetic changes with dysplasia. In total, four of 10 lesions were graded as mild dysplasia while there seemed a more or less identical number of allelic losses in these samples as compared to the lesions graded as moderate or severe dysplasia. This seems to be in contrast to earlier results as we previously noticed a correlation between the grade of dysplasia and the number of allelic losses (36), but it is likely because of the selection of the markers. Losses on chromosomes 3p, 9p, and 17p belong to the earliest in malignant progression and consequently do not differ between the various pre-cancerous lesions. There was a significant association between the grade of dysplasia and the number of MLPA changes, most likely as the number of MLPA markers is larger, and more chromosomes are measured that in part are related to late progression.

As per definition, the LOH assay detected genetic alterations in all pre-cancerous fields but not in normal oral mucosa and exfoliated cell samples from non-cancer controls. It should be noted that LOH marker D11S1883 was not evaluable in all 10 paraffin-embedded resection margins because of the length of the amplicon (240–259 bp). It is well known that DNA isolated from formalin-fixed paraffin-embedded specimens is fragmented and therefore is difficult to analyze by markers larger than 200 bp. However, in the 20 fresh non-invasive samples from exfoliated cells tested, this marker has proven to work well (data not shown). We investigated, using normal blood DNA of a non-cancer control subject informative for all markers, whether the cut-off values limiting the 99% confidence interval of our select marker panel are within the generally accepted values of less than 0.5 and greater than 2.0. At present, we did not adapt these cut-off values. The polymorphic nature of the microsatellite markers does not allow fine-tuning of the cut-off values on the repeat analysis of normal DNA of only one single control subject. This will require large-scale screening studies with many repetitive mucosal samples in control subjects. Compared with the MLPA assay, the LOH assay is more robust, with the feasibility for automatization. Nevertheless, the assay remains laborious and in principle necessitates that normal DNA is analyzed in parallel as reference.

The DNA index assay has been used on the tissue samples according to an established protocol based on ICM (27, 28). From the 10 analyzed pre-cancerous fields, only four showed an aberrant DNA content while they all contained (per definition) genetic changes by LOH measurement. Moreover, the six analyzed corresponding tumors were all aneuploid while in three of these six sample pairs, the fields were diploid. There are several technical and biological explanations for this finding. We cannot formally exclude that we missed aneuploid cells by limitations in sensitivity of the assay. Both FCM and automated image analyses can be exploited to assess the DNA index, but we used the more sensitive ICM method, and the chance that we missed aneuploid cells is therefore relatively low. We can also not formally exclude that the insufficient enrichment of the pre-neoplastic cells in this assay might have played a role. The area of interest in the samples was enriched by macroscopic removal of the peripheral tissue while for the LOH and MLPA analysis, tissue was enriched by dissection under the microscope. On the other hand, measurement of aneuploidy was more sensitive than LOH and MLPA analysis as at least one aneuploid cell in 10 normal cells could be detected, which counterbalances the different level of enrichment in the comparison of the different assays. Furthermore, despite the fact that all precursor fields contained genetic changes as per definition, these alterations are often minor encompassing one or two chromosomes, and this might not result in a change in the DNA index that can be detected by ploidy analysis. Hence, the most logical explanation in our view is not technically, but biologically and we should consider that aneuploidy is a specific and reliable marker for late progression. DNA index measurement might therefore be more suited for risk assessment of an identified pre-cancerous field, and of less value for early diagnosis despite the higher analytical sensitivity. Unexpectedly, ploidy status seemed to be unrelated to dysplasia grading as three of four aneuploid pre-cancerous fields were graded as mild dysplasia. In contrast, five of six diploid margins showed moderate to severe dysplasia.

Early diagnosis of oral pre-cancerous fields might be of importance for clinical management, particularly in high risk populations. Patients who are known to be at high risk for developing OSCC and would qualify for screening are those with visible oral pre-neoplastic lesions, those who have been treated for OSCC, those with excessive tobacco and alcohol abuse, and those who are genetically pre-disposed such as Fanconi anaemia patients. A study containing a large number of subjects having a pre-cancerous field as determined by the genetic analysis of tissue biopsies and in subjects without risk factors (young age, non-smokers) will be necessary to determine the clinical sensitivity and specificity of the assays, respectively.

In conclusion, the LOH assay might be a valuable screening tool to detect the presence of oral pre-cancerous fields, particularly in high-risk patients. Whether the DNA ploidy assay adds to this approach to follow risk for progression remains to be determined.