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Prospective, blinded comparison of cytology and DNA-image cytometry of brush biopsies for early detection of oral malignancy

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SUMMARY

Objectives: Adjunctive techniques like DNA image cytometry (DNA-ICM) have been attributed to enhance the diagnostic performance of oral brush biopsies. The aim of the study was an evaluation of brush biopsies, analysed according to morphological criteria and by DNA-ICM vs. histological findings in a blinded prospective trial.
Materials and methods: Eighty eight brush biopsies of 70 patients were sampled. Only clinical suspicious but not evident malignant oral lesions were included. Clinical diagnosis was leukoplakia ($n = 36$), lichen planus ($n = 18$), verruciform erythroplakia ($n = 12$), erythroleukoplakia ($n = 9$), erosion ($n = 7$) and induration ($n = 6$). Evaluation was conducted via histology, cytology and DNA-ICM.
Results: Histological diagnosis revealed eight cases of squamous intraepithelial dysplasia (SIN 1 $n = 6$, SIN 2 $n = 2$), four cases of carcinoma-in situ and 25 cases of oral T1-cancer. Remaining cases were leukoplakia ($n = 28$), lichen planus ($n = 15$) and local inflammation ($n = 8$). Brush biopsy detected malignant lesions including SIN > 1 with a sensitivity of 55% and a specificity of 100%. DNA-ICM had a sensitivity of 70% and a specificity of 100%. The combination of both methods showed a sensitivity of 76% and a specificity of 100%. The predominant reason for false negative results were sampling errors with insufficient cells (86% in brush biopsy and 100% in DNA-ICM).
Conclusion: DNA-ICM has the potential to substantially improve the sensitivity of a pure morphological interpretation of oral brush biopsies. Method inherent sampling errors may be accountable for a lower sensitivity compared to conventional histological diagnosis. Therefore, DNA-ICM should not be used to rule out malignancy, when lesions are already clinically suspicious for oral cancer.

Introduction

Oral and oropharyngeal squamous cell carcinoma (OSCC) is one of the most common malignancies reported worldwide and is a significant cause of cancer deaths. Despite aggressive multimodal therapy, the 5-year survival rate has remained less than 50% for advanced stages of OSCC, whereas, when discovered at earlier stages, the survival rate may approximate or even exceed 80%.[1,2] Therefore, detection strategies to identify small tumors or even precursor lesions may improve clinical outcome[3] in terms of survival and post treatment quality of life.[1,4] It is generally accepted that the vast majority of oral cancers arise from clinically distinct precursor lesions and that neoplastic transformation typically takes months or even years.[5] Regarding an estimated worldwide prevalence of 2% for leukoplakias which represent the most common "risk" lesions with annual overall transformation rates ranging from 0.7% to 2% in various populations and geographical areas,[6,7] surveillance of precursor lesions is an ongoing challenge in many healthcare systems. Despite tremendous progress in the understanding of basic mechanisms involved in malignant transformation and substantial improvements in molecular diagnostics, there is no single marker or a panel of markers available yet, that allow for reliable prediction of malignant transformation for the individual patient.[8]

Dentists and general practitioners are frequently the first contact in the healthcare system for patients with oral mucosal alterations. However, discrimination of benign and potentially malignant lesions may be difficult by visual examination, resulting in considerable false negative or false positive rates at initial clinical

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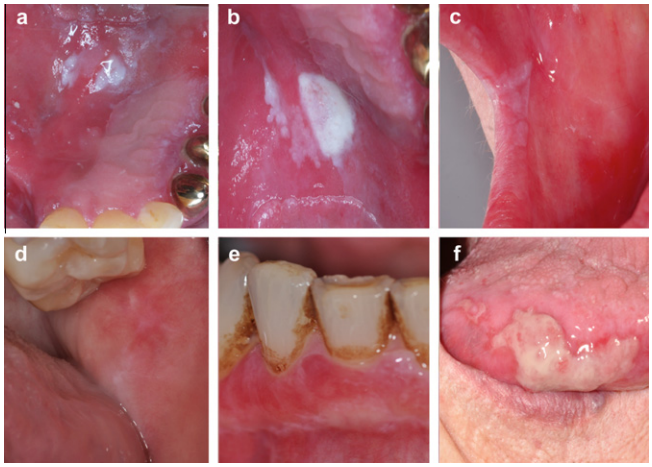

Figure 1 Representative images of oral risk lesions that were enclosed in the study. The respective results of the clinical, histological as well as cytological examinations are given in below table.

Figure	Clinical diagnosis	Histology	Cytology	DNA-ICM
1a	Leukoplakia	SIN1	Negative	Polyploid
1b	Leukoplakia	Leukoplakia	Negative	Diploid
1c	Leukoplakia	Lichen planus	Negative	Diploid
1d	Lichen planus	Carcinoma	Negative	Aneuploid
1e	Erythro-leukoplakia	SIN2	Suspicious	Aneuploid
1f	Erosion	Inflammation	Negative	Diploid

judgement.[9] Surgical biopsy followed by histopathology – the diagnostic gold standard – is invasive and time consuming; it may not be carried out by dentists or general practitioners and may not be possible in anxious as well as incompliant patients with asymptomatic lesions.[10,11] Therefore, multiple adjunctive techniques have been introduced to support clinicians in the assessment and management of suspicious oral mucosal lesions. These techniques are based on visual (such as *in vivo* fluorescence[12] or toluidine blue staining[13]) as well as cytological principles (for example brush biopsy,[14] immune cytology[15] and gene expression analysis[16]).

Overall, evidence on diagnostic properties is rare, rendering all these methods a source of controversy. Regarding oral brush biopsies, which is one widespread diagnostic tool, pure morphological analysis has been supplemented by several additional techniques (for example DNA-image-cytometry[17] and Oral CDX[18]) in the last years.

For the combination of morphological cytology and DNA-image-cytometry, which is the focus of this communication, results in terms of 97.8% sensitivity and 100% specificity have been reported.[18] However, these results were derived from a cohort which included even advanced stages of oral squamous cell carcinomas and the cytological diagnosis was not blinded against clinical and histological data.

Therefore, it was the aim of this communication to report on morphological cytological analysis and DNA-image-cytometry-results obtained on clinically suspicious but not obvious malignant lesions, fully blinded against the histological results.

Materials and methods

Patients

From 01/2005 to 01/2009, 70 patients (45 male, 25 female; mean age 62 years (range 27–88)) with 88 oral lesions of uncertain dignity were included in the study (Fig. 1a–f). The number of lesions per patient in this cohort was as follows: one patient had four suspicious sites, four patients had three sites, seven patients had two sites and the remaining 58 patients had one site. Lesions with typical neoplastic appearance already clinically classified as carcinomas were excluded. In brief, excluding criteria were ulceration, bleeding, indurated raised margins with exophytic and/or papillary growth, deep infiltrations in the surrounding tissue as well as fungating tumors. The protocol of the study was approved by the local ethic committee (No. 837.233.08(6230)) of Rhineland-Palate and all individuals provided written informed consent for study participation. The patients were examined at the Department of Oral-, Maxillofacial and Plastic Surgery at the University Medical Center Mainz in accordance with the protocol and in compliance with the moral, ethical, and scientific principles governing clinical research as set out in the Declaration of Helsinki.[19] The initial clinical diagnoses were leukoplakia ($n = 36$; 41%), lichen planus ($n = 18$; 20%), verruciform erythroplakia ($n = 12$; 14%), erythroleukoplakia ($n = 9$; 10%), erosion ($n = 7$; 8%) and burrowing, submucosal induration ($n = 6$; 7%). The findings were located on tongue ($n = 26$; 29.5%), floor of the mouth ($n = 18$; 20.5%), alveolar ridge ($n = 18$; 20.5%), mucosa of the cheek ($n = 10$; 11.4%), palate ($n = 9$; 10.2%), vestibule ($n = 3$; 3.4%), lip ($n = 3$; 3.4%) and on the retromolar area ($n = 1$; 1.1%).

Brush biopsy and incisional biopsy

From each lesion, four slides from a separate brush biopsy were taken without prior mucosal disinfection. A proprietary brush (Cytobrush ®Plus GT; Medscan, Malmo, Sweden) was used to obtain epithelial cells. The small brush was rotated for 10 revolutions over an approximately 10×10 mm (100 mm^2) area, resulting in most cases in petechial bleeding points. Immediately after harvesting, the cells were transferred onto four pre-charged glass microscope slide (SuperFrost®Plus, Menzel, Braunschweig, Germany) in

Table 1
Categories of cytological diagnoses for brush biopsy interpretation in our study setting.

Insufficient	No cells to be evaluated
Tumor cell negative	Unsuspicious epithelial cells; reactive, inflammatory or regenerative changes; mild dysplasia
Suspicious for tumor cells	Sparse abnormal epithelial cells; moderate and severe dysplasia
Tumor cell positive	Unequivocal malignant epithelial cells

a rotating manner and fixed by ethyl alcohol (Merckofix®, Merck, Darmstadt, Germany). One slide was sent to the local Department of Pathology for hematoxilin–eosin (H&E) staining and cytopathologic interpretation. Two slides were used for DNA-ICM examination after conventional Feulgen staining (protocol included in supplement) by two independent investigators. The fourth slide remained unstained for reserve.

After harvesting cells, a scalpel incision biopsy (approximately 0.1 × 0.1 cm) was taken from exact the same area. The obtained tissue was formalin-fixed, paraffin-embedded and diagnosed at the Department of Pathology.

Morphological analysis was conducted at the Deparment of Pathology, DNA-ICM at the Department of Otorhinolaryngology and the Department of Oral-, Maxillofacial and Plastic Surgery. All investigators were blinded against the results of the complementary method and against the histological results which served as the gold standard. For each of the analysis method, the same protocols were followed.

Histology

The paraffin-embedded specimens were cut into 3–4 μm thick sections and stained with hematoxilin–eosin (H&E). The respective thickness and the staining are standard in our clinical practice. The tissue was classified as normal (optionally with inflammatory alterations), dysplastic (subdivided in squamous intraepithelial neoplasia (SIN) 1 and >1[20]), or invasive carcinoma. The definition of invasive carcinoma was based on the detection of infiltrative growth patterns for individual malignant cells or cell clusters.

Cytology

After H&E-stain, the smear was examined for tumor cells or suspicious cells, respectively, according to generally accepted cytopathologic criteria[21,22] (Table 1) by two experienced pathologists (BS, BG).

DNA-ICM

A computerized image analysis system was used for DNA measurements (AutoCyte QUIC-DNA Workstation; AutoCyte, Elon College, NC, USA). The measurements were analyzed with AutoCyte QUIC-DNA software after Feulgen staining.[23,24] Applying 566-nm monochromatic light, the staining intensity after the Feulgen reaction correlates to the DNA content of the cell, allowing ploidy analysis.[25] For each specimen, 250–300 randomly chosen cells of the suspicious cell population were measured. The mean DNA content of >30 non-neoplastic nuclei (lymphocytes) was taken as internal reference. Coefficients of variation of reference cells were below 5%. Reference extinction was normalized to the 2c (2-fold DNA content)-value of the reference cells. The standards and guidelines of the European Society for Analytical Cellular Pathology (ESACP) for DNA-ICM were followed.[26–29] All DNA-ICM measurements were conducted in duplicates by two investigators (SM, KPW) with experience in the method.[30]

Table 2
Criteria for the diagnostic interpretation of DNA-histograms.[17,31,32].

DNA-diploid	STL between 1.80c and 2.20c
DNA-polyploid	STL between 1.80c and 2.20c and STL between 3.60c and 4.40c
DNA-aneuploid	STL outside the ranges of DNA-diploid and DNA-polyploid distribution patterns and/or events >9c
	STL = DNA stemline
	1c = DNA-content of a single chromosomal set

Assessment of classification criteria of DNA-ICM

As described before,[17,31,32] a slide was classified as **DNA-diploid**, if only one DNA stemline (STL) between 1.80c and 2.20c was detected. **DNA-polyploidy** was assumed, if there were DNA-STLs between 1.80c and 2.20c and between 3.60c and 4.40c. A lesion was characterized as **DNA-aneuploid**, if an abnormal DNA-STL <1.80c and >2.20c or <3.60c and >4.40c and/or 9c exceeding events were detected (Table 2). As described before, a DNA-STL was defined as the G_0/G_1 phase fraction of a proliferating cell population.[17] DNA-ICM was conducted without knowledge of the nature of the lesion or the histopathological grading.

Re-evaluation

In cases of false-negative or contradictory results for histology, cytology and DNA-ICM, respective slides were re-assessed by three experienced persons (BG, BS, KPW). If there was an disagreement, the respective slides were reviewed together until a mutual agreement was obtained. For screening of sampling or screening errors[33,34] the same three persons reviewed the cytology and DNA-ICM specimens. The prior unstained slide was stained with H&E and included in cytology examinations at this time. In cases of sampling errors, the smears did not contain abnormal cells. This may result either from deficient harvesting of cells or from loss of cells within the processing of the slides. Screening errors may result out of a subjective interpretation error by the cytopathologist.

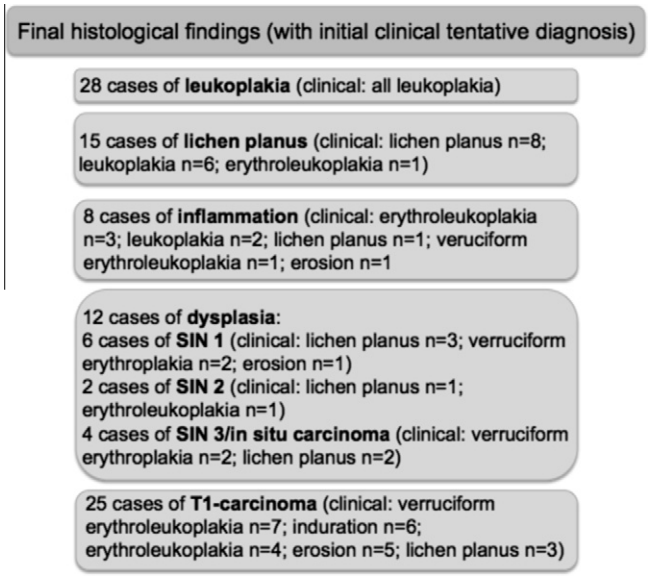

Figure 2 Diagnostic matrix showing histological results together with the respective initial tentative clinical diagnoses.

Table 3
Sensitivity and specificity as well as positive and negative predictive value of cytology vs. histology in detection of malignancy (n = 88 lesions). Overall sensitivity was 55% (CI: 0.38–0.71). Sensitivity after excluding sampling errors: 89% (CI: 0.69–0.97). Specificity: 100% (CI: 0.94–1).

		Histology	
		Benign (including SIN = 1)	Malignant (including SIN > 1)
Cytology, all cases	Negative for tumor cells (including SIN = 1)	57	14
	Positive for tumor cells (including SIN > 1)	0	17
Cytology, cases with sampling error excluded	Negative for tumor cells (including SIN = 1)	57	2
	Positive for tumor cells (including SIN > 1)	0	17

Table 4
Sensitivity and specificity as well as positive and negative predictive value of DNA-ICM vs. histology in detection of malignancy (n = 87 lesions). Overall sensitivity was 70% (CI: 0.52–0.83). Sensitivity after excluding sampling errors: 100% (CI: 85–1). Specificity: 100% (CI: 0.94–1).

		Histology	
		Benign (including SIN = 1)	Malignant (including SIN > 1)
DNA-ICM, all cases	Diploid and polyploid	57	9
	Aneuploid	0	21
DNA-ICM, cases with sampling error excluded	Diploid and polyploid	57	0
	Aneuploid	0	21

Statistical analysis

The analyses were conducted using SPSS Statistics version 20 for Macintosh (IBM, Armonk, NY, USA). Data were expressed as median values with ranges (minimum–maximum) and as percentages.

For the analysis of sensitivity, specificity positive predictive value and negative predictive value, respectively, the following grouping of diagnosis was performed: The histological diagnoses of the scalpel biopsies were grouped as "negative" in cases with benign changes or with the finding of mild dysplastic epithelial cells (SIN 1) only,[17,20,35] and as "positive" if cells with moderate or severe dysplasia (SIN 2, SIN 3) or malignant tumor cells were present.[17] In parallel, grouping for cytopathology was performed. For DNA-ICM, a case was classified as "negative" if DNA-aneuploidy could not be proven, and as "positive" for the finding of DNA-aneuploidy. For the measurements of sensitivity and specificity, confidence intervals were included.

Results

Histology

On histological evaluation, 51 lesions were classified as reactive or inflammatory in nature, showing neither malignancy nor epithelial dysplasia. The remaining specimens showed invasive squamous cell carcinomas (25 cases), epithelial dysplasia (12 cases; SIN 1 n = 6, SIN 2 n = 2, SIN 3/in situ carcinoma n = 4). The histopathological diagnoses together with the initial tentative clinical diagnoses are summarized in Fig. 2. After histological confirmation, the respective cases of epithelial dysplasia and squamous cell carcinoma received surgical treatment via resection of the neoplasms. If lymph node involvement was suspected by means of palpation, sonography and/or CT analysis, additional neck dissections were conducted.

Cytology

The cytologic analysis of each slide of the brush biopsies was able to detect 17/31 of the malignant or high-risk lesions (SCC and epithelial dysplasia including SIN > 1). All low risk lesions (unspecific and SIN $\leqslant$ 1) (57/57) were classified correctly (table 4). This adds up to a sensitivity of 55% (95% confidence interval (CI): 0.38–0.71) and a specificity of 100% (CI: 0.94–1) as compared to histology. The positive predictive value (PPV) is 100%, the negative predictive value (NPV) 80% (Table 3).

DNA-ICM

DNA-ICM could be performed in 87 samples. One sample (histology: malignant) with an insufficient cell yield was excluded. 60 samples were classified as diploid, 6 as polyploid and 21 as aneuploid. The detection of malignant or high risk lesions (malignancy including SIN > 1) was accomplished in 21/30 cases. Again, all low risk lesions (reactive or inflammatory) were diagnosed correctly (57/57; sensitivity: 70% (CI: 0.52–0.83); specificity: 100% (CI: 0.94–1); PPV: 100%; NPV: 86%; Table 4).

The 6 polyploid slides were from 2 carcinomas, 2 cases of mild dysplasia (SIN 1) and 2 cases of histologically negative lesions. Thus, the finding of polyploidy could not contribute to the finding of a correct diagnosis in this small group of patients.

Between the two investigators, no differences in the results were observed.

Cytology and DNA-ICM

The combination of cytology and DNA-ICM was considered as positive if one of the methods showed positive results. Hereby, 23/30 high-risk lesions (including SIN > 1) and 57/57 low risk lesions could be classified correctly (sensitivity: 77% (CI: 0.59–0.88); specificity: 100% (CI: 0.94–1); PPV: 100%; NPV: 89%; Table 5).

Re-evaluation

Cytology

The 14 false-negative slides of the brush biopsies were examined. A sampling error was seen in 12/14 cases (86%; no suspicious cells at all n = 6, inflammation n = 5, SIN 1 n = 1). In these cases malignant cells could not be found in the slides even after re-evaluation.

In 2/14 cases (14%), a screening error was detected. In these cases re-evaluation lead to the detection of previously unrecognized malignant cells.

Table 5
Sensitivity and specificity as well as positive and negative predictive value of cytology supplemented by DNA-ICM vs. histology in detection of malignancy ($n = 87$ lesions). Sensitivity for all cases: 77% (CI: 0.59–0.88). Sensitivity after exclusion of sampling errors: 100% (CI: 0.85–1), Specificity: 100% (CI: 0.94–1).

		Histology	
		Benign (including SIN = 1)	Malignant (including SIN > 1)
Cytology and DNA-ICM, all cases	Negative for tumor cells (negative/SIN 1) and DNA-diploid or DNA-polyploid	57	7
	Positive for tumor cells (>SIN 1) or DNA-aneuploid	0	23
Cytology and DNA-ICM, cases with sampling error excluded	Negative for tumor cells (negative/SIN 1) and DNA-diploid or DNA-polyploid	57	0
	Positive for tumor cells (>SIN 1) or DNA-aneuploid	0	21

DNA-ICM

The 9 false-negative were examined again and sampling errors were seen in all cases.

Discussion

The oral brush biopsy is a meanwhile well established and non-invasive method for analysis of oral lesions, though its diagnostic value via conventional cytology is seen controversely. Inconsistencies and potential bias of prior studies regarding oral brush biopsies have been reported by several authors.[36,37] For example, in the study of Sciubba and coworkers, the gold standard histological examination was performed in patients with suspicious lesions only. For the large group of subjects with innocuous lesions, no incision biopsy was conducted. Accordingly, critical information to assess the brush cytology was lost.[38,39] Whereas Babshet and coworkers considered brush cytology to be as good as histopathology,[10] Driemel et al. showed a sensitivity of 79% in comparison to conventional biopsy.[40] In a similar study, Koch et al. stated a sensitivity of 88.5% for identifying oral squamous cell carcinoma.[41] In all these studies, clinical obvious oral carcinomas were included which typically cause a bias towards higher sensitivity.

DNA-image-cytometry (DNA-ICM) could be an adjunctive technique to increase detection rates of high-risk precursor lesions or oral cancer (OC).[42–44] Accordingly, the detection of aneuploidy, which is the basis of DNA-ICM, has been claimed to be one of the most sensitive and effective indicators of malignant transformation in the oral cavity[45,46] and has also been suggested to be an important predictor of malignant potential[47] even when an oral lesion is classified as benign according to morphological criteria of histology. This has been attributed to the concept that cells with an aneuploid DNA pattern are resembling cancer at a single cell level and can therefore be defined as potentially malignant.[48] Therefore, we examined brush cytology, DNA-ICM and the combination of brush cytology/DNA-ICM in detecting malignancy in oral lesions of clinical uncertain dignity.

In our study, in all patients – from exactly the same site as the brush biopsy was taken – an appropriate incisional biopsy was conducted and histological examination was set as gold standard. Nevertheless, even for experienced pathologist examiners, diagnosis of oral epithelial dysplasia grades remains challenging and even histological diagnosis may be incorrect in some cases; poor interobserver reproducibility for assessing oral premalignant lesions and dysplasia was described before.[49–51] This may interfere with the interpretation of cytologic and DNA-ICM results. Accordingly, we conducted a second appraisal of the 12 dysplastic lesions.

In our fully blinded clinical setting, for the exclusion of high-risk precursor lesions and carcinomas, cytology achieved an only limited sensitivity in OC diagnosis of lesions of clinically uncertain dignity. It has to be kept in mind that previous examinations with higher sensitivity rates[31,36,42] also involved clearly-defined entities such as large oral squamous cell carcinomas[52] that were excluded in our study. A reduction of sensitivity when observing smaller malignant lesions by brush cytology has been described by our group before.[12] Additionally, one slide was provided for cytologic interpretation as used in normal clinical routine. As sampling errors were detected in most of the false-negative samples, the used method of brush biopsy may not be appropriate to obtain complete transepithelial specimens. For example, in cases of hyperkeratotic lesions, non-respresentative though prominent epithelial layers may be primary sampled. Accordingly, more aggressive brush designs, such as for the OralCDx system,[39] in order to yield cells from deeper layers of the epithelium may be needed. This is in controversy to Böcking and coworkers which stated that transepithelial sampling may not be required to diagnose dysplasia because malignant cells will migrate from basal to superficial layers.[11] From our data and from the results of other groups considering detection of early "high-risk" dysplasias it can be estimated that the DNA-ICM is a highly valuable tool if sampling errors can be ruled out.[11,31,53] Examination of more than two slides from the same lesion with cytology and DNA-ICM could also be taken into consideration.

Combining brush cytology and DNA-ICM, a total increase in sensitivity and specificity of oral brush biopsy up to 100% has been reported.[17,31] In these studies, DNA-ICM was only applied if at least dysplasia was seen in prior cytology. We conducted DNA-ICM on all slides to obtain comparable data. However, the prior reported data have not been obtained in a blinded study design and have not been confirmed by other research groups. In our blinded study we could show that DNA-ICM has a lower sensitivity compared to the data cited above. Though, when eliminating sampling errors, the DNA-ICM method achieved a comparable high sensitivity and specificity rate. However, although the post hoc elimination of sampling errors confirms the methodological integrity of the pure DNA-ICM evaluation process, the overall performance of the diagnostic process ("brushing cells and analysing them by DNA-ICM") remains crucial. From the perspective of a clinician it is of minor relevance, whether 30% false negative results can be secondarily attributed to sampling errors or are inherent to the method of evaluation since he cannot distinguish between different sources of uncertainty without taking a biopsy for histological analysis. It merits further research efforts to explore, whether the shortcomings of sampling deficiencies may be overcome in the near future. But even with the brush-proprietary errors, DNA-ICM still achieves a reasonable accuracy and can improve the performance of morphological analysis.

Altogether, these results are in accordance to up to date cervical cancer screening examinations via cytologic testing and DNA-ICM.[54] It should be mentioned that 1% of the slides used for DNA-ICM were quantitatively or qualitatively insufficient. This problem has been described earlier,[55] but could easily be solved by a repeated brush biopsy.

Conclusion

Brush cytology and DNA-ICM are fast, non-invasive and low-cost methods to examine suspect oral lesions. Although we could not confirm the extraordinary high accuracy of others after primary examination, our blinded study supports the principal value of DNA-ICM-evaluation to improve the accuracy of morphological cytological analysis. However, due to the severe problem of sampling errors in early and hyperkeratotic lesions, even DNA-ICM analysis should not be regarded as a confirmatory method to rule out malignancy but rather as an adjunct to improve the quality of brush biopsy as a screening instrument.

Role of the funding source

No funding was obtained.

Conflict of interest statement

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2012.12.006.

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