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Cytologic and DNA-cytometric very early diagnosis of oral cancer

D. Maraki[1], J. Becker[1], A. Boecking[2]

[1]*Department of Oral Surgery and* [2]*Institute of Cytopathology, Heinrich Heine University, Duesseldorf, Germany*

BACKGROUND: The aim of this study was to evaluate the diagnostic accuracy of exfoliative cytology (EC) and DNA-image cytometry applied to suspicious oral lesions compared with synchronous histology.

METHODS: Brush- and scalpel biopsies were obtained from 98 patients with suspicious oral lesions. In cases, in which EC revealed malignant or suspicious cells, nuclear DNA-contents were measured using a TV image analysis system.

RESULTS: Among 98 oral lesions both cytological and histological diagnosis showed no sign of malignancy or dysplasia in 75. In 23 cases cytology yielded tumor cell-positive (15), suspicious (four) or doubtful (four) results. DNA-cytometry showed aneuploidy in 19 of these. The comparison between cytological diagnosis combined with DNA-cytometry and biopsy-histology resulted in a sensitivity of 100% and a specificity of 97.4%.

CONCLUSION: In conclusion, cytology with DNA-cytometry is a highly sensitive, specific and non-invasive method for the early diagnosis of oral epithelial neoplasia, showing excellent compliance among patients.

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Keywords: DNA-aneuploidy; DNA-image cytometry; oral exfoliative cytology; oral mucosal lesions

Introduction

White patches of the oral cavity, which cannot be scraped off and can neither be clinically nor pathologically attributed to any other disease, defined as leukoplakia are considered to be precancerous lesions with a certain potential of developing a squamous cell carcinoma (1–5). Leukoplakia is the most common premalignant lesion of the oral cavity, but not the only one. Except leukoplakia also erythroplakia, lichen planus, and actinic keratosis (caused through ultraviolet radiation of sunlight) are considered to be premalignant lesions for oral squamous cell carcinoma (1). Moreover, red lesions (erythroplakia), which are ulcerated and bleeding, with (leukoerythroplakia) or without white components are considered to signify the presence of severe epithelial dysplasia or *in situ* or invasive carcinoma (6–8). Unfortunately, the 5-year survival rate of patients with distant metastases at the time of the first diagnosis is only 19%, whereas for operable tumors in an early, localized stage it approximates 80% (7). Yet, it is very difficult to achieve an early diagnosis, due to the unreliability of visual oral examination (9).

The high morbidity and mortality rates of oral squamous cell carcinoma in western countries (6) lead to an increased necessity of its early diagnosis and treatment. Until now, scalpel biopsy has been the only reliable and accepted method for the examination and diagnosis of suspicious oral mucosal lesions, although inter- and intraobserver variability of histological diagnoses yielded insufficient results (10–12). It is also well-known that most dentists are reluctant to refer patients to a scalpel biopsy (13). Nowadays, an alternative method for the examination of suspicious oral mucosal lesions is exfoliative cytology (EC). It is principally based on the method of Papanicolaou, which is accepted worldwide, as a successful method in order to screen for epithelial dysplasias, *in situ* or invasive carcinomas of the uteri cervix. Additionally, a tool adjuvant to the cytological diagnosis of oral mucosal smears: DNA-image cytometry has been recently introduced for the very early diagnosis of malignant transformation of squamous epithelial cells (13–15). This is used to detect the cytometric equivalent of chromosomal aneuploidy, which is called DNA-aneuploidy (16). After Feulgen restaining of the same slides used for cytological diagnosis, the cytometric equivalent of chromosomal aneuploidy can be taken from DNA-cytometry (17). DNA-aneuploidy is internationally accepted as a marker for the neoplastic transformation of cells (16, 18, 19).

The aim of this prospective clinical study was to evaluate the diagnostic accuracy of EC taken from ulcerated, white or red spotted and suspicious lesions of the oral mucosa. Additionally, the accuracy of DNA-image cytometry as an adjuvant diagnostic tool was determined. The 'golden standard' were histological

Correspondence: Dimitra Maraki, Department of Oral Surgery, Heinrich Heine University, Moorenstr. 5, D-40225 Duesseldorf, Germany. Tel.: 0049 211 8118149, Fax: 0049 211 8116550. E-mail: dimitra.m@tiscali.de
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diagnoses of the same lesions. Our hypothesis is that EC combined with DNA-image cytometry can detect malignant cells prior to the histological diagnosis.

Materials and method

Clinical procedure

The study population consisted of 111 patients at the beginning of our study, but only 98 met the criteria for inclusion we had defined; the patients who were included in this study, showed clinically suspicious oral lesions, in which dysplasia or neoplasia could not be ruled out. Thirteen patients were excluded; two because they were unwilling to undergo a scalpel biopsy, five because the lesions, which clinically were suspicious for dysplasia turned out to have a traumatic cause and have healed before we could take a scalpel biopsy and six because they were lost for follow up (see Appendix for flow chart). Brush biopsies were taken from 98 oral lesions of the 98 patients mentioned above, who were examined between February 2002 and January 2004 in the Department of Oral Surgery, University of Duesseldorf, Germany. From the respective oral lesions also scalpel biopsies were taken for histological diagnosis and none of these were excised *in toto* at the time of first examination. The mean age of the population was 61 years (range 25–87), it consisted of 54% females and 46% males. The final diagnoses were: 15 squamous cell carcinomas, 21 leukoplakias, three erythroplakias and 59 other, inflammatory oral lesions (Table 1). For follow up we divided the patients, which showed no dysplasia or neoplasia in the final cytological and histological diagnosis into two groups: those who had precancerous lesions (leukoplakia, erythroplakia, actinic keratosis, lichen planus) and those who did not have such lesions. Both groups underwent a follow up of 4–6 months (mean time 5 months) after the first examination; the first group (precancerous lesions) was controlled with clinical and cytological examination, whereas the second one only with clinical examination. The patients showing dysplasia or neoplasia in the brush or scalpel biopsy underwent surgical treatment and a clinical and cytological follow up was performed 4–6 months later. Finally, patients showing dysplasia in EC or DNA-aneuploidy in DNA-image cytometry, but no sign of dysplasia in scalpel biopsy underwent a follow up of 4 months.

Before brush biopsies of the suspicious lesions were performed, every patient underwent a clinical examination of the oral cavity and the medical history was documented. To obtain a smear we used a Cytobrush cell collector (Cytobrush GT, Med-Scand Medical, Malmo, Sweden) (20), which was rolled at the same place of the mucosal lesion at least five times with gentle pressure. The brush was turned around its own axis on four different positions of a glass slide in order to transfer the cells, which were immediately fixed with Merckofix-spray (Merck, Darmstadt, Germany) (Fig. 1). After the obtainment of smears, we have also taken excisional biopsies of the respective oral lesions. The examination of the slides and the biopsy specimens were carried out in the Institute of Cytopathology and in the Clinic of Dermatology, University of Duesseldorf, Germany respectively.

Staining of the smears

The glass slides were stained according to Papanicolaou and examined according to accepted cytological criteria for dysplasia and malignancy (21). Boecking (22) has defined the following categories of cytological diagnoses: 'insufficient' for specimens without any or with exclusively autolytic cells, 'tumor cell-negative' (**1**) for inconspicuous, reactive or inflammatory cellular images, 'doubtful for tumor cells' (**2**) in cases with slight atypical cellular changes (e.g. with mild or moderate dysplasia), 'suspicious for tumor cells' (**3**) if only sparse abnormal or severe dysplastic cells were seen or the diagnostic criteria for malignancy were only vague and 'tumor

Table 1 Diagnoses of 98 oral mucosal lesions[a]

Final diagnosis	*Number (n)*	*Cytological diagnosis*	*DNA-distribution*	*Histological diagnosis*
Lichen planus	37	(**1**) In $n = 35$	–	No dysplasia
		(**2**) In $n = 2$	DNA-polyploidy = 2	
Pemphigoid/gingivitis desquamativa	17	(**1**) In $n = 17$	–	No dysplasia
Linear IgA disease	1	(**2**) In $n = 1$	DNA-polyploidy = 1	No dysplasia
Aphthous ulcers	1	(**1**) In $n = 1$	–	No dysplasia
Asthma spray stomatitis	2	(**1**) In $n = 2$	–	No dysplasia
Actinic keratosis	1	(**2**) In $n = 1$	DNA-polyploidy = 1	Mild dysplasia (negative)
Leukoplakia	21	(**1**) In $n = 20$	–	No dysplasia
		(**3**) In $n = 1$	DNA-aneuploidy = 1	Severe dysplasia (positive)
Erythroplakia	3	(**3**) In $n = 3$	DNA-aneuploidy = 3	Severe dysplasia (positive) in $n = 2$ (in $n = 1$ after 12 months) Mild to severe dysplasia (positive) in $n = 1$ (four pathologists)
Squamous cell carcinoma	15	(**4**) In $n = 15$	DNA-aneuploidy = 15	Squamous cell carcinoma ($n = 15$)

[a]Explanation of diagnostic categories of cytological diagnoses in Table 2.

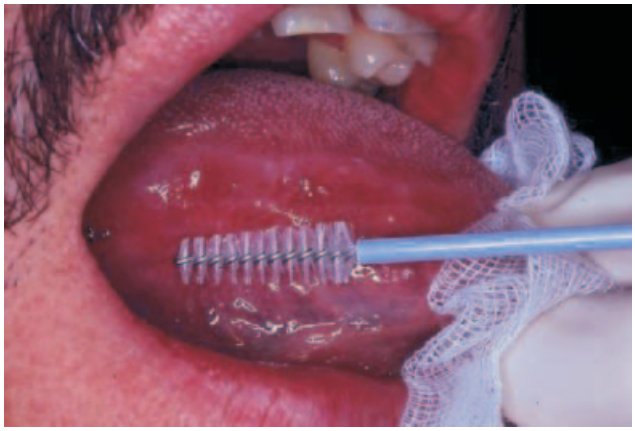

Figure 1 Obtaining an exfoliative smear.

cell-positive' (**4**) for smears containing unequivocal malignant cells (Table 2). In cases of a doubtful, suspicious (**2** and **3**) or tumor cell-positive (**4**) cytological diagnosis, also the nuclear DNA-contents of the respective cells were measured after Feulgen restaining of the slides, using a TV image analysis system. For that purpose the slides were uncovered in xylene, destained and restained with Schiff's reagent (18, 23–25). If necessary, restaining of Feulgen-stained slides according to Papanicolaou was possible.

Measurement of DNA-contents

The AutoCyte QUIC DNA-workstation (AutoCyte, Burlington, NC, USA/Zeiss, Jena, Germany) was used for the measurements of the nuclear DNA-contents in the Feulgen-stained slides; it consists of a conventional light microscope adapted to a TV black-white camera of a computer-based TV image analysis system (26). The European Society for Analytical Cellular Pathology (ESACP) task force on standardization of diagnostic DNA-image cytometry (18, 24, 27) has defined standards for the performance of these systems.

A lesion has been classified as DNA-diploid, if there was only one DNA stemline (STL) between 1.80c and 2.20c. A lesion was characterized as DNA-polyploid if there were DNA-STLs between 1.80c and 2.20c and between 3.60c and 4.40c. DNA-aneuploidy was assumed if there were abnormal STLs <1.80c and >2.20c or <3.60c and >4.40c and/or 9c exceeding events (9cEE) >0 (Table 3) (28). A DNA-STL was defined as the G0/G1 cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region) (18, 29).

Table 2 Categories of cytological diagnoses (22)

Insufficient	(–)
Tumor cell-negative	(**1**)
Doubtful for tumor cells	(**2**)
Suspicious for tumor cells	(**3**)
Tumor cell-positive	(**4**)

Table 3 Criteria for the diagnostic interpretation of DNA-histograms[a] (28)

DNA-diploid	STL $>$ 1.80c $<$ 2.20c
DNA-polyploid	STL $>$ 1.80c $<$ 2.20c and $>$3.60c $<$ 4.40c
DNA-aneuploid	STL $<$ 1.80c $>$ 2.20c or $<$3.60c $>$ 4.40c and/or events $>$9c

[a]STL, DNA stemline; 1c, DNA-content of a single chromosomal set.

Statistical method

Sensitivity and specificity were calculated according to the four-field table (30). The 'gold standard' for the evaluation of cytologic/DNA-cytometric diagnoses were the histological diagnoses obtained on scalpel biopsy of the lesions, considering positivity and negativity for each test. Cytologically negative were non-neoplastic, mild and moderate dysplastic cells, whereas cytologically positive were severe dysplastic or malignant cells. DNA-cytometric negative diagnoses were DNA-euploid, whereas positive diagnoses DNA-aneuploid histograms.

Specificity was defined as the probability of the tested method (EC) to recognize correctly healthy patients (negative for severe dysplasia or neoplasia) as such. Sensitivity was defined as the probability of the tested method (EC) to recognize sick patients (positive for severe dysplasia or neoplasia) as such. False positive diagnoses were those in which cytology stated severe dysplasia or cancer cells, or DNA-image cytometry DNA-aneuploidy but no malignancy was found by histology.

Results

In 75 of the 98 exfoliative smears the cytological diagnoses revealed no presence of malignant or dysplastic cells. Twenty-three (23) cases were cytological diagnosed as doubtful (actinic keratosis: $n = 1$, lichen planus: $n = 2$, linear IgA disease: $n = 1$), suspicious (leukoplakia: $n = 1$, erythroplakia: $n = 3$) or positive for tumor cells (squamous cell carcinomas: $n = 15$). DNA-image cytometry revealed DNA-aneuploidy in 19 of these 23 cases (Table 1).

Table 1 also shows that 35 of 37 oral lesions with the diagnosis of lichen planus, 20 of the 21 leukoplakic lesions and 22 other inflammatory lesions of the oral mucosa cytological revealed no signs of malignant transformation, which was in agreement with the histological diagnoses. Furthermore, in four cases (linear IgA disease = 1, lichen planus = 2, and actinic keratosis = 1; Fig. 2) the cytological diagnosis was doubtful for tumor cells; because of this fact, DNA-image cytometry was carried out, which showed DNA-polyploidy (Fig. 3). This was in accordance with the histological diagnoses (negative for dysplasia $n = 3$ and mild dysplasia $n = 1$ respectively). None of the cases mentioned above, which were negative for severe dysplasia and neoplasia at the time of the first cytological and histological examination, showed any suspicion of malignancy in the follow up, performed 4–6 months later.

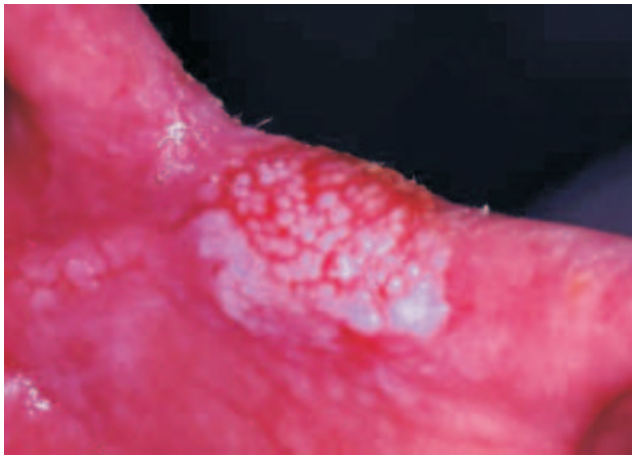

Figure 2 Actinic keratosis of the lip.

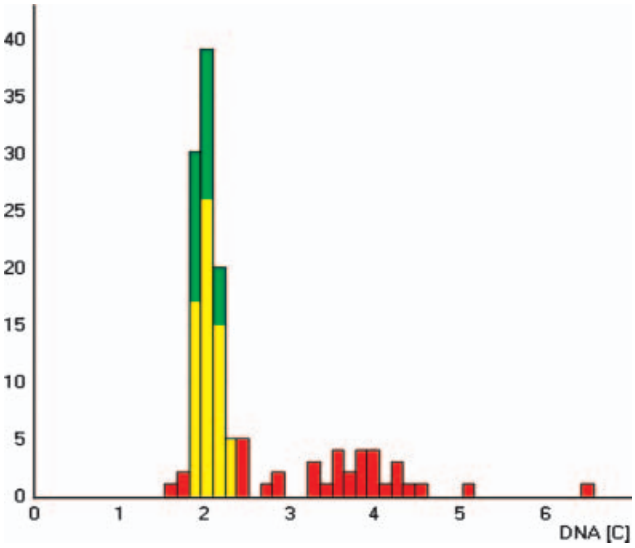

Figure 3 DNA-histogram of an actinic keratosis, revealing DNA-polyploidy.

One case with the clinical diagnosis of erythroplakia showed in both cytological and histological diagnosis severe dysplasia and DNA-cytometry revealed aneuploidy. In one erythroplakic case cytology showed severe dysplasia and DNA-cytometry aneuploidy, whereas discrepancies occurred concerning the histological diagnoses of four pathologists, ranging from mild to severe dysplasia. A follow up of this patient is still being expected, because we consider this case to represent an early cytological diagnosis of malignancy obtained by DNA-cytometry prior to the histological diagnosis. In a further leukoplakic case the first histological diagnosis was negative for dysplasia, whereas cytology showed severe dysplasia and DNA-cytometry aneuploidy. Because of this fact the histological specimen was further examined and finally severe dysplasia has been revealed. The last case suspicious for tumor cells was an erythroplakia in the soft palate (Fig. 4). Cytology revealed severe dysplasia and DNA-cytometry DNA-aneuploidy (Fig. 5), whereas the histological diagnosis was only mild dysplasia. This patient developed a

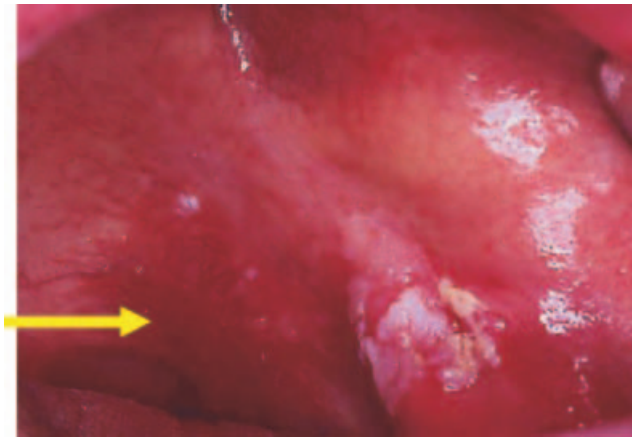

Figure 4 Erythroplakia of the soft palate.

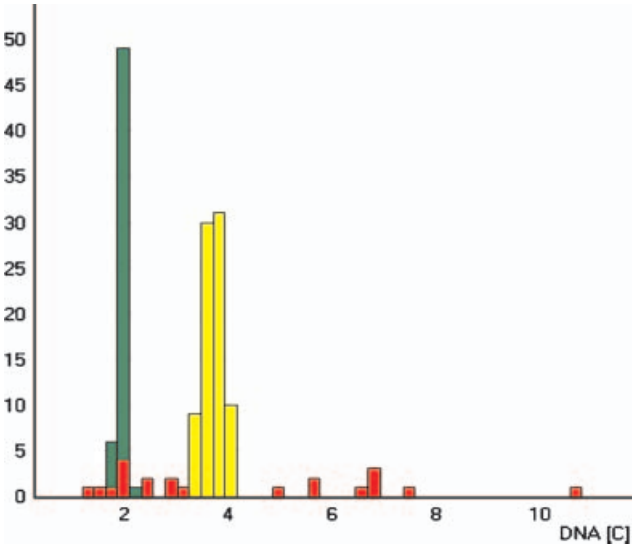

Figure 5 DNA-histogram of the erythroplakic case, revealing DNA-aneuploidy [stem lines (STLs) at 4c and 6c, 9c exceeding events (9cEE) = 4].

carcinoma *in situ* 1 year later. The last two cases are typical examples of an early diagnosis of cytology combined with DNA-cytometry of *in situ* squamous cell carcinomas of the oral cavity prior to the histological diagnosis.

Moreover, the presence of malignant cells was proven in 15 lesions with DNA-aneuploidy, which was in accordance with the clinical and histological diagnoses (squamous cell carcinomas). Figure 6 shows the clinical view of a squamous cell carcinoma.

The clinical and cytological follow up of all patients showing severe dysplasia or neoplasia after surgical treatment was negative for malignancy.

Statistical analysis

According to the comparison of the cytological diagnoses combined with DNA-image cytometry and the histological diagnoses, the specificity of cytology/cytometry for the detection of histological non-neoplastic

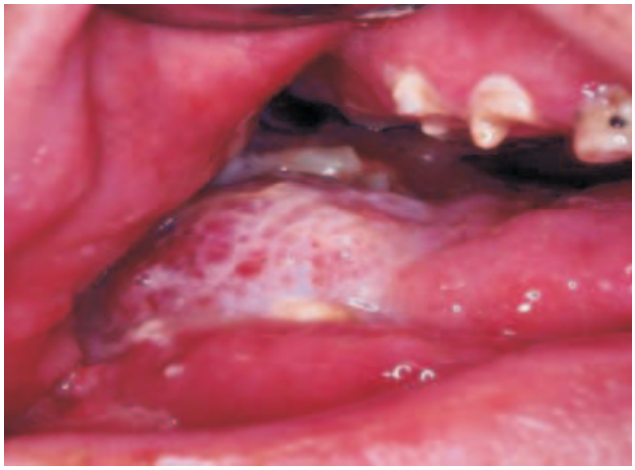

Figure 6 Squamous cell carcinoma.

Table 4 Sensitivity and specificity of cytology combined with DNA-image cytometry vs. histology ($n = 98$ cases)

	Histology	
Cytology/DNA-cytometry	*Benign (including moderate dysplasia)*	*Malignant (including severe dysplasia)*
Negative for tumor cells (negative/doubtful and DNA-euploid)	79	0
Positive for tumor cells (suspicious positive and DNA-aneuploid)	2	17

tissue was 97.4% and its sensitivity for the detection of histological proven cancer was 100% (Table 4).

Discussion

Using cytology and DNA-image cytometry, we were able to prove that oral lesions with the diagnosis of lichen planus and other inflammatory diseases, have shown no suspicious cells. A recent review of the literature places the rate of malignant transformation of lichen planus to squamous cell carcinoma at 0.2% (1). The significance of the absence of malignant cells in the non-cancerous lesions examined in this study is high, because with the help of EC we were able to exclude severe dysplasia or neoplasia in these lesions, which clinically have raised suspicion for malignancy. Furthermore, EC offers the possibility for an early diagnosis of severe epithelial dysplasia or neoplasia prior to histological diagnosis. Thus, tumor cell-negative cytological diagnoses can exclude the probability of a malignant transformation of the respective lesions in the follow up period reported in this study. A question arising from these results is the potential use of EC on clinically normal mucosa in high-risk patients as heavy smokers, in order to predict a possible malignant transformation. We do not recommend such unfocused brush biopsies as, so far, there is no evidence that oral cancer evolves in macroscopically normal oral mucosa.

On the contrary, the presence of malignant cells was proven in one of 21 leukoplakic cases (4.76%), in all erythroplakic cases and in all squamous cell carcinomas. A review of 2236 leukoplakic cases from five studies has revealed a range of malignant transformation of leukoplakia between 2.2 and 17.5% (2). Furthermore, Sciubba (7), Silverman et al. (31) and Mashberg et al. (8) emphasized the fact that erythroplakia, occurring as either an isolated lesion or as a component of leukoplakia (erythroleukoplakia) has been repeatedly proven to be a marker of severe epithelial dysplasia or carcinoma *in situ*. In fact, 90% of erythroplakic lesions were histological diagnosed as *in situ* or invasive carcinomas (1). Based on our results and those of the authors mentioned above, we propose brush biopsies with cytological/DNA-cytometric examination for microscopic evaluation of white or red patches of the oral cavity (leukoplakias or erythroplakias). The finding of tumor cells or DNA-aneuploidy should lead to a total excision of the respective lesions and histological examination.

Although early diagnosis of squamous cell carcinoma plays the most important role for the increase of the survival rate of patients, literature shows that most carcinomas are being diagnosed, when the patient has already displayed evidence of spread to regional lymph nodes and distant metastases. This fact leads to 5-year survival rates under 50% (7, 9, 13). Unfortunately, sensitivity of cytological diagnosis in 1306 cases from 14 studies (32) showed an average of only 87.4% ranging from 73.8 to 100%. This could explain the fact, which until now histological examination remained the 'golden standard' for diagnosis and identification of malignant oral lesions.

Within the limits of our present study, we have shown that sensitivity of cytological diagnosis combined with DNA-image cytometry may reach 100%, whereas specificity was 97.4%. Considering that in one erythroplakic case the intraobserver variability among four pathologists led to results ranging from mild to severe dysplasia and because of the cytological and DNA-cytometric diagnosis (severe dysplasia with DNA-aneuploidy), we suppose that this case represents an early cytological and DNA-cytometric diagnosis of malignancy prior to the histological diagnosis. Therefore, the specificity of our present study could reach 100%, if presence of malignancy will be proven by the intended follow up of this patient. After cytological and histological examination of 158 oral mucosal lesions, Remmerbach et al. (13) have proven that sensitivity of cytological diagnosis combined with DNA-image cytometry was 98.2% and specificity 100%, when compared with the 'golden standard' of histology. These facts lead to the conclusion that the possibility of overseeing the malignant potential using EC combined with DNA-image cytometry is very low, ranging from 0 (in our study) to 1.8% (13). On the opposite, the examinations of Sudbo et al. (33) on archived material have shown that the nuclear DNA-content in cells of oral leukoplakia can be used to predict the risk of oral epithelial dysplasias up to 5 years before histological

confirmation. In this study, among 150 patients with histological verified epithelial dysplasia, 36 developed squamous cell carcinoma. DNA-cytometry showed in 105 patients DNA-diploidy, in 20 DNA-polyploidy and in 25 patients DNA-aneuploidy at the time of the initial diagnosis. A carcinoma developed in only three of the 105 diploid lesions when compared with 21 of the 25 aneuploid lesions. Remmerbach et al. (14) proved in the clinical setting that DNA-aneuploidy might predict histological obvious malignancy 1–15 months prior to histology. Also in the clinical setting of our study, we were able to show that EC combined with DNA-image cytometry can predict malignant transformation up to 1 year before its histological confirmation.

DNA-image cytometry has repeatedly been used as an adjuvant diagnostic tool, in order to detect DNA-aneuploidy in oral epithelial lesions. DNA-image cytometry is indicated, in order to clarify the prospective biological behavior of mild and moderate epithelial dysplasias and in order to verify the diagnosis in tumor cell-positive cases (13, 19). In order to reach a high detection rate of DNA-aneuploidy, both abnormal DNA-STLs and rare 9cEE are being used as algorithms. According to Remmerbach (13), no DNA-cytometric assessment of cytological tumor cell-negative cases is necessary, because they hardly ever reveal DNA-aneuploidy.

In conclusion, EC in combination with DNA-image cytometry is a very sensitive, highly specific, inexpensive and non-invasive diagnostic tool, which shows a very good acceptance among patients. It may be used for the non-invasive investigation of even large clinically suspicious oral mucosal lesions in order to early detect oral cancer and its recurrence and to specify the prospective behavior of oral dysplasias.

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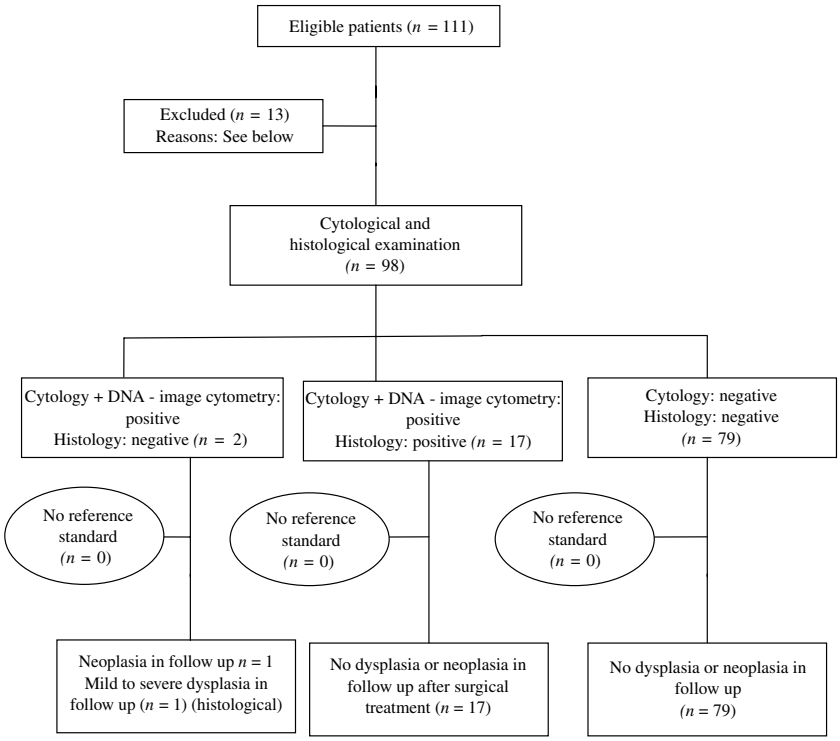
Appendix: Flow chart



Reasons to exclude patients:
- refused scalpel biopsy ($n = 2$)
- the suspicious fordysplasia lesions had a traumatic causeand healed before we could take a biopsy ($n = 5$)
- lost for follow up ($n = 6$)