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Cytologic and DNA-cytometric examination of oral lesions in lichen planus

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BACKROUND: The aim of the present study was to evaluate the diagnostic value of exfoliative cytology (EC) and DNA image cytometry applied to oral lesions of lichen planus (LP; $n = 56$), in order to detect or exclude malignant transformation.
METHODS: Brush and excisional biopsies were obtained from 56 patients. In cases of oral LP in which brush biopsies were suspicious for tumor cells, nuclear DNA contents were measured, using a TV Image Analysis System.
RESULTS: In 50 patients EC yielded tumor cell-negative, doubtful in four cases and suspicious results obtained in two cases. DNA image cytometry revealed DNA-aneuploidy only in the two suspicious cases. The comparison between cytologic/DNA-cytometric diagnosis and biopsy histology resulted in a total agreement (LP without dysplasia: 54 and squamous cell carcinoma in LP: two cases).
CONCLUSIONS: In conclusion, cytology with DNA-cytometry is a highly sensitive, specific, and non-invasive method, which can be used for periodical follow up of oral LP lesions in order to early detect or exclude malignancy.
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Keywords: DNA image cytometry; lichen planus; oral cancer; oral exfoliative cytology

Introduction

Immunologic diseases of the oral mucosa often manifest as desquamative gingivitis (DG), which is recognized to be a clinical variation of a number of disorders ranging from vesiculobullous to idiopathic diseases, but not a distinct pathologic entity (1–3). The epithelium is quite friable and can be removed, leaving a red surface that bleeds easily (1, 4). The majority of DG cases are manifestations of immunologic diseases, such as lichen planus (LP), mucous membrane pemphigoid, pemphigus vulgaris, linear immunoglobulin A (IgA) disease (4).

Lichen ruber planus is a mucocutaneous disease of unknown cause, which manifests at the oral mucosa as a reticular, erosive (ulcerative), plaque, papular, and atrophic form (5). DG arises especially at the erosive and atrophic forms of oral LP and can be the only or initial sign of oral involvement in approximately 25% of these patients (2). A subject of great interest has been the potentially malignant nature of oral LP. Although, many studies were performed in order to specify the malignant potential of oral LP, this topic remains controversial (6–11). In particular, some authors suggested that squamous cell carcinomas most frequently arose from the erosive, plaque, and atrophic types (12–16). Other studies doubted the histologic diagnoses of LP lesions with dysplastic features, which seemingly transformed to squamous cell carcinomas (6).

Until now, scalpel biopsy has been the only reliable and accepted method for the definite diagnosis of oral manifestations of immunologic diseases and for the detection of epithelial dysplasia. Exfoliative cytology (EC) has been recommended for the examination of oral pre-cancerous and cancerous lesions, but less is known on the potential of this technique to examine superficial erosions or ulcerations, i.e. in patients with DG.

Nowadays, a tool adjuvant to the cytologic diagnosis of oral mucosal smears is DNA image cytometry, which has been introduced for the very early diagnosis of malignant transformation of squamous epithelial cells (17–21). After Feulgen restaining of the same slides used for cytologic diagnosis (22), the DNA content of about 300 diagnostically relevant epithelial cells is established by measuring their integrated optical density (IOD; = amount of dye per nucleus) after stochiometric Feulgen staining. This can interactively be performed using a TV Image Analysis System combined with a conventional light microscope. Thirty normal epithelial cell-nuclei are taken as internal reference cells. Whereas normal or reactive squamous cells reveal euploid (diploid or polyploid) DNA contents, malignant cells

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mostly show aneuploid patterns. DNA-aneuploidy is internationally accepted as marker of neoplastic cell transformation, which is the cytometric equivalent of chromosomal aneuploidy (23–26).

The aim of the present study was to evaluate the diagnostic capabilities of EC combined with DNA image cytometry applied to patients with inflammatory oral lesions of LP, concerning the exclusion or detection of malignancy by exclusion or confirmation of DNA-aneuploidy.

Materials and methods

Clinical procedure

The study population initially consisted of 58 patients, which revealed oral lesions suspicious for LP. Two of them were excluded, because they refused to have a second scalpel biopsy after the first one was inconclusive. The mean age of the population was 58 years (range: 23–76); it consisted of 72% females and 28% males. Before brush and excisional biopsies of the suspicious lesions were performed, every patient underwent a clinical examination and photo-documentation of the oral lesions and the medical history was documented.

Brush biopsies were taken as a routine examination from 56 oral lesions of the buccal mucosa, attached gingiva or tongue of 56 patients with suspicion of LP (erosive, atrophic or in combination with the reticular type), who were examined between January 2002 and August 2005 in the department of oral surgery, University of Düsseldorf, Germany. After brush biopsy, excisional biopsies were taken from the same areas in these patients for routine histopathologic (fixation in buffered formalin) and immunohistologic examinations (deep frozen in liquid nitrogen). For immunohistochemistry cryostat sections of the specimens were examined through routine direct immunofluorescence (DIF), using antibodies against IgG and IgA (for details see Ref. 27).

To obtain a smear we used a Cytobrush cell collector (Cytobrush GT, Med-Scand Medical, Malmo, Sweden; 28), which was rolled at the same place of the mucosal lesion at least five times with gentle pressure (Fig. 1). 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). 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 Düsseldorf, Germany respectively.

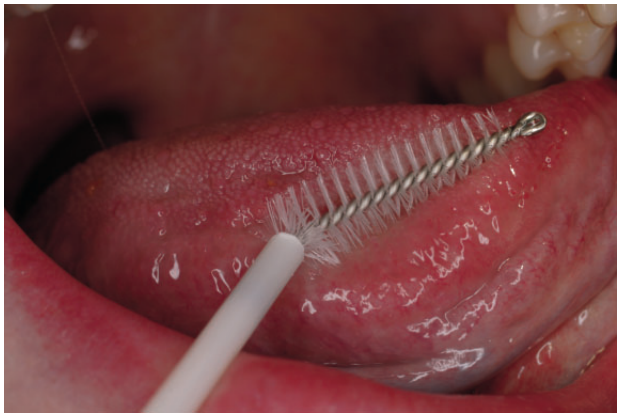

Figure 1 Obtaining a brush biopsy.

Staining of smears

The glass slides were stained according to Papanicolaou and examined applying internationally accepted cytologic criteria for squamous epithelial cells (29). Boecking (30) has defined the following categories of cytologic diagnoses: 'insufficient' for specimens without any or with exclusively autolytic cells; 'tumor cell-negative' (i) for inconspicuous, reactive or inflammatory cellular images; 'doubtful for tumor cells' (ii) in cases with slight atypical cellular changes (e.g. abnormal regenerative cells, mild or moderate dysplasia); 'suspicious for tumor cells' (iii) if only sparce abnormal or severe dysplastic cells were seen or the diagnostic criteria for malignancy were only vague and 'tumor cell-positive' (iv) for smears containing unequivocal malignant cells. In cases with a doubtful, suspicious (2, 3) or tumor cell-positive (4) cytologic 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 (26, 31–33). If necessary, restaining of Feulgen-stained slides according to Papanicolaou was possible.

Measurement of DNA contents

The nuclear integrated optical density (IOD) is taken as cytometric equivalent of the nuclear DNA contents measured in Feulgen-stained slides after internal calibration with normal squamous epithelial cells ($n = 30$). The AutoCyte QUIC DNA-workstation (AutoCyte, Burlington, NC, USA; Zeiss, Jena, Germany) was used for the measurements; it consists of a conventional light microscope adapted to a TV black and white camera and a computer-based TV Image Analysis System (34, 35). The European Society for Analytical Cellular Pathology (ESACP) task force on standardization of diagnostic DNA image cytometry (26, 31, 36) has defined standards for the performance of these systems. A minimum of 300 randomly selected dysplastic or malignant epithelial cells per specimen was measured at random. Otherwise, only the available atypical or tumor cells were measured, whereas specimens with < 50 diagnostically relevant cells were considered insufficient. Thirty cytologic normal epithelial cells were measured in each of these specimens as an internal reference to establish the normal 2c value. The coefficient of variation (CVs) of reference cells was always below 5% and no correction factor was applied (26, 31).

The diagnostic criteria and classification of the lesions are shown in Table 1 (37, 38). A DNA-stemline 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; 26, 39).

Table 1 Criteria for the diagnostic interpretation of DNA histograms[a] (38)

DNA-diploid	STL: >1.8c <2.2c
DNA-polyploid	STL: >1.8c <2.2c and >3.6c <4.4c, 9cEE = 0
DNA-aneuploid	STL: <1.8c >2.2c or <3.6c >4.4c and/or events >9c

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

Results

Among 56 exfoliative smears, 50 revealed no signs of dysplastic or malignant cells, whereas four lesions were diagnosed as doubtful for tumor cells (Fig. 2), revealing regenerative epithelial cells (Fig. 3) and two were suspicious for malignancy (Fig. 4), showing severe epithelial dysplasia. DNA image cytometry showed DNA-polyploidy (Fig. 5) in the doubtful four cases and DNA-aneuploidy in the suspicious cases (Fig. 6).

The histologic specimens of all patients showed the typical findings of oral LP (stratified epithelium, inflammatory subepithelial infiltrate, occasionally sawtooth-like appearance of the epithelium; Fig. 7). DIF revealed no IgG or IgA autoantibodies.

Cytologic/DNA-cytometric diagnoses were in total agreement with the histologic diagnoses, confirming the diagnosis of oral cancer in two cases. Both of these patients came in our department for a routine examination and treatment after an incisional biopsy of a suspicious area was taken from a private practitioner 4 months before. According to the description of the patients, oral LP was present for at least 3 years, but neither of them could define the precise period of time. In the first case, the incisional biopsy from the buccal mucosa performed in December 2004, confirmed the diagnosis of LP and revealed no dysplasia or malignancy. The incisional biopsy from the lateral border of the tongue (Fig. 4) of the second patient, performed in March 2005, was compatible with LP revealing mild dysplasia.

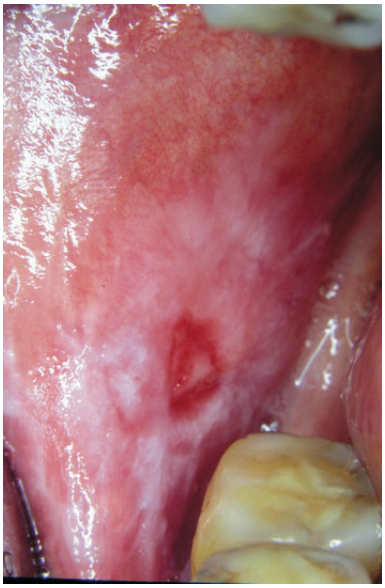

Figure 2 Reticular and erosive oral lichen planus on the buccal mucosa.

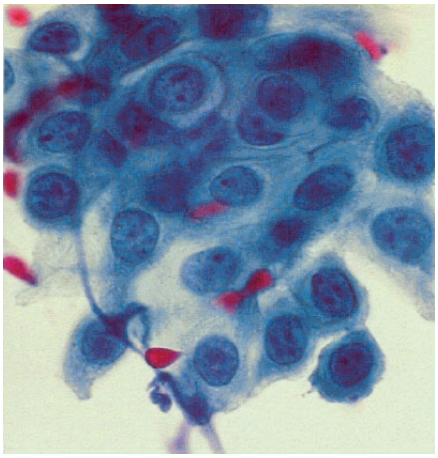

Figure 3 Cytologic specimen showing abnormal regenerative epithelial cells of a lichen planus lesion (1000×).

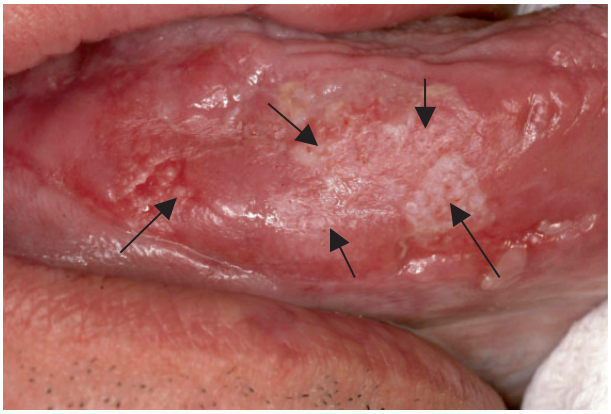

Figure 4 Histologically proven invasive squamous cell carcinoma of the lateral border of the tongue of a lichen planus patient, revealing erosive, atrophic, and reticular areas. The arrows indicate the region of the incisional biopsy.

Because of the positive cytologic/DNA-cytometric diagnosis, we decided to perform new incisional biopsies of the same lesions in both cases, which finally revealed an invasive squamous cell carcinoma (Fig. 8a,b,c).

Discussion

Using EC and DNA image cytometry, Remmerbach et al. (19, 20) were able to show not only a very high sensitivity (98.2%) and specificity (100%) of EC combined with DNA image cytometry, but also its potential for an early diagnosis of oral cancer (1–15 months prior to histologic confirmation). In our previous studies, we could show three cases of a very early diagnosis of oral cancer in comparison with synchronous histology and also high rates of sensitivity (100%) and specificity (100%) of the method (17, 18). Furthermore, Sudbø et al. (40, 41) could show on archived material that 84% of DNA-aneuploid oral leukoplakias and 92% of DNA-aneulpoid erythroplakias developed malignant transformation after a

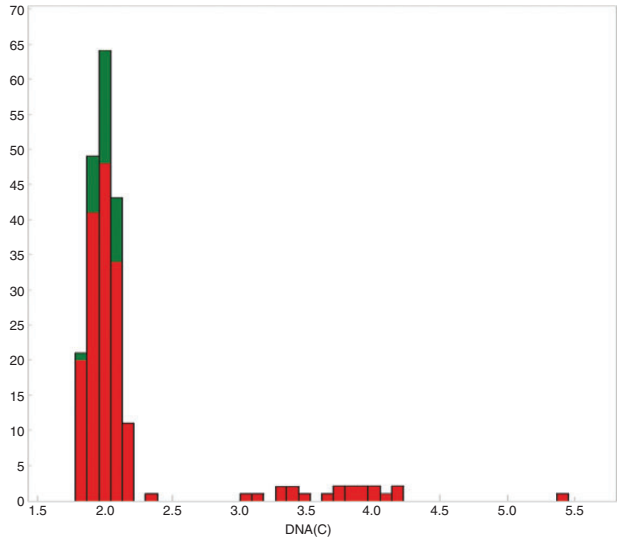

Figure 5 DNA polyploid histogram of the lesion illustrated in Fig. 2 with stemlines at 2c and 4c. The green columns show DNA contents of reference cells, whereas the red ones of squamous epithelial cells.

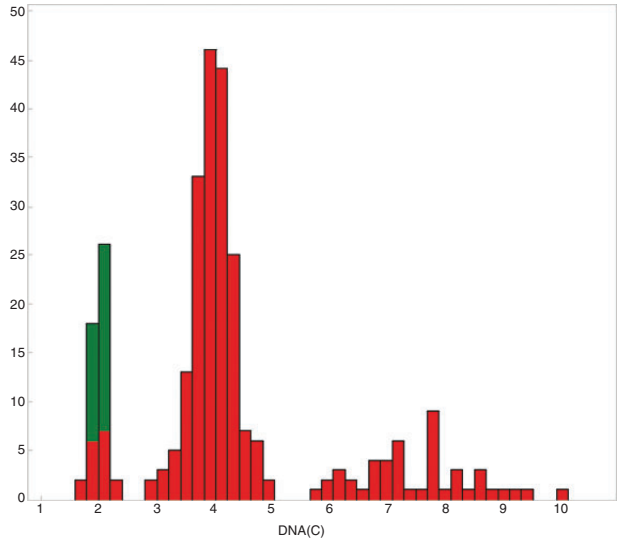

Figure 6 Peritetraploid aneuploid DNA histogram of the lesion in Fig. 4 (dominant DNA-stemline around 4c, a second stemline around 7.5c, and four cells with DNA content >9c).

mean time of 35 months and 53 months respectively following initial histologic examination.

In the present study, we have shown that two of 56 patients (3%) with oral LP revealed oral cancer, on the contrary some authors claim this disease not to show malignant transformation. According to Holmstrup et al. (42) and Holmstrup (43) oral LP fulfils the World Health Organization criteria of a potentially malignant condition, whereas Eisenberg and Krutchkoff (6) have doubted its relationship to oral cancer. In particular, Eisenberg and Krutchkoff (6) introduced the term 'lichenoid dysplasia', in order to define oral pre-cancerous lesions with lichenoid features, resembling LP. They also criticized that some studies describing malignant transformation of oral LP lesions were poorly documented, providing insufficient clinical and histopathologic features for the diagnosis of oral LP. In order to avoid false diagnoses of LP and to ensure the validity of our clinical study, we performed incisional biopsies both for histopathologic examination and DIF and we discussed the clinical features and subjective complaints of the patients with the Department of Dermatopathology, which also evaluated the histologic specimens. In particular, our clinical diagnosis concerning the exclusion of lichenoid reactions and supporting the suspicion of LP lesions was mainly based on the following facts: (i) exclusion of any causative factors for lichenoid reactions in our study population, e.g. relevant medication, metal restorations, etc.; and (ii) clinical diagnosis of LP only in cases, where the lesions were symmetrical or almost symmetrical on both sides of the mouth, showing typical characteristics of the disease, as interlacing white keratotic lines or striae with a central erosion, sometimes covered with fibrin or showing peripheral atrophy or a combination of the above, in some cases also combined with DG.

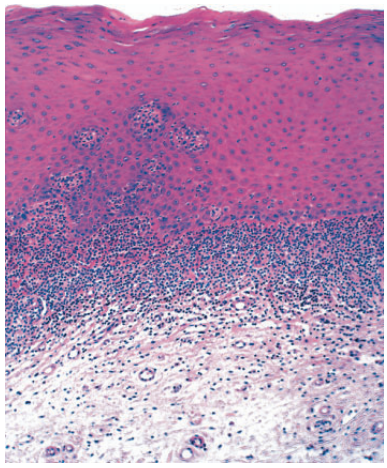

Figure 7 Histologic specimen of a lichen planus patient showing no dysplasia or malignancy (200×).

Based on our clinical experience and on the cytologic and histopathologic findings, we support the opinion of Holmstrup et al. (42) that erosive and atrophic oral LP lesions include the possibility of malignant transformation. Considering the cases of malignant transformation of oral LP mentioned above, we emphasize the possibility of sampling errors, resulting from the fact that incisional biopsies can only cover a relatively small area of oral lesions, in comparison with brush biopsies, which enable the screening of large mucosal areas. In spite of the facts mentioned above, it is of great importance that in our study, in the two cases of squamous cell carcinoma, the malignancy co-existed with the LP lesions in the time that the patients came to our department for the first time. Co-existence is only an indication of LP malignant transformation. In order to be able to provide sufficient proof, long-term follow-up studies are necessary.

Therefore, we suppose that EC combined with DNA image cytometry is a suitable diagnostic method for a long-term follow-up of oral LP or lichenoid lesions because of its non-invasiveness and high acceptance in all our patients, in order to exclude or early identify

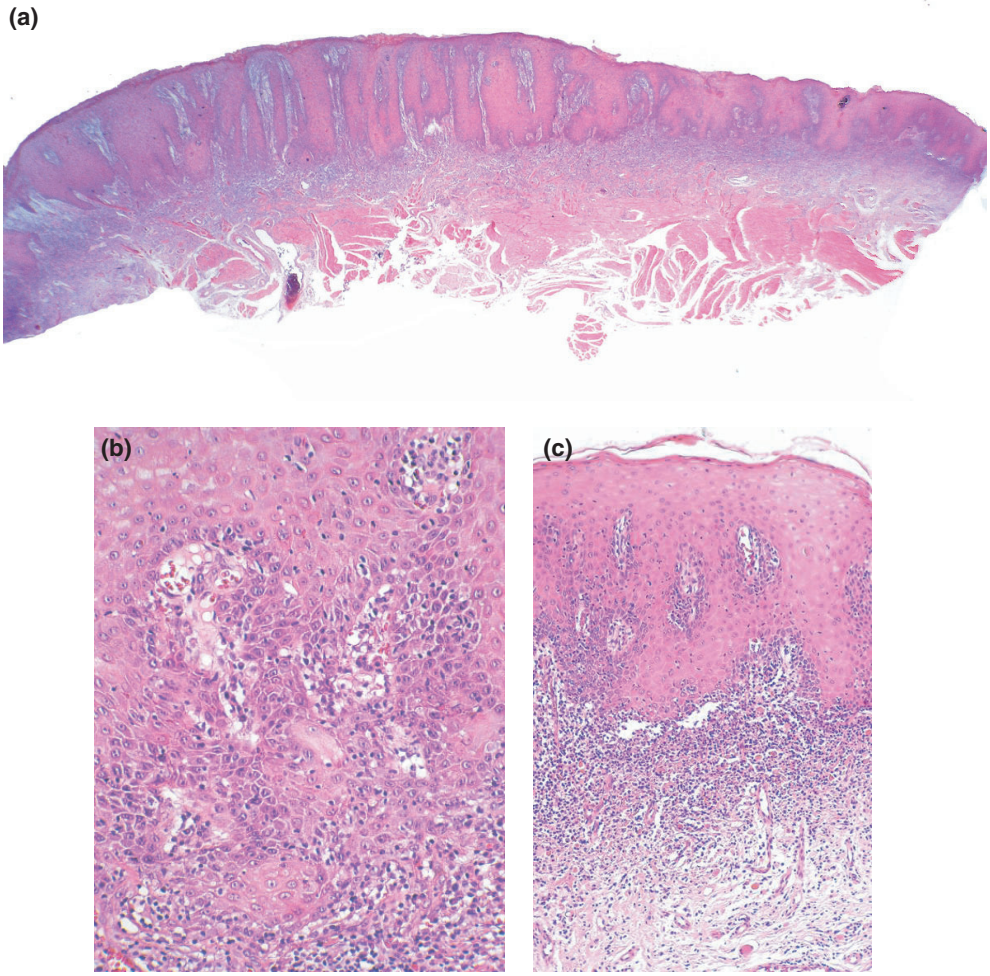

Figure 8 (a) Photomicrograph revealing well differentiated squamous cell carcinoma of a specimen with typical signs of lichen planus (20×). (b) Magnification (200×) of the squamous cell carcinoma region of the specimen in (a). (c) Magnification (200×) of the lichen planus region of the specimen in (a).

malignant transformation avoiding the invasiveness, limited reproducibility, and sampling error of histologic diagnosis. However, a long period of clinical, cytologic, and histologic follow-up is demanded, in order to provide sufficient information on this topic. Our hypothesis is that, because of the problems associated with the histologic diagnosis of oral immunologic diseases and especially of oral LP, EC combined with DNA-cytometry might be an appropriate, non-invasive, and safe diagnostic tool for an accurate monitoring of these lesions concerning the exclusion or confirmation of malignancy. Furthermore, it is our opinion that more work should be carried out to determine phenotypical and genotypical changes in oral lesions, in order to be sure that the results justify the conclusions drawn.

Within the limits of our present study, we conclude that EC combined with DNA image cytometry is a highly specific, sensitive, inexpensive, and non-invasive diagnostic tool compared with excisional biopsies, in order to exclude or confirm malignant transformation of oral lesions, which showed very good acceptance among our patients and allowed the screening of larger mucosal areas. It may therefore be recommended for a non-invasive periodical control of oral inflammatory, erosive lesions in patients with LP, especially in those with risk factors for the development of malignant epithelial tumors in the oral cavity (i.e. tobacco and alcohol consumption), replacing excisional biopsies.

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