

Immunohistochemical study on topoisomerase II α , Ki-67 and cytokeratin-19 in oral lichen planus lesions

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Abstract Oral lichen planus (OLP) is a chronic mucocutaneous inflammatory disease defined as a precancerous condition. We determined the expression patterns of proliferation markers topoisomerase II α (topo II α) and Ki-67 and an intermediated filament protein cytokeratin-19 (CK-19) in atrophic OLP. These markers were selected because our recent microarray analysis indicated they might aid in identification of potentially malignant lesions. The expression patterns were correlated with the DNA content of these lesions shown to be useful in detection lesions at risk for malignant transformation of OLP. A series of 81 formalin-fixed, paraffin-embedded biopsies from 70 patients suffering from atrophic OLP were immunostained with monoclonal antibodies against topo II α , Ki-67 and CK-19 using standard methods. Of the 70 patients, there were eight patients who had dysplastic changes in OLP lesions. During the follow-up, altogether five patients got cancer in the OLP area even though no dysplastic changes were present in the preceding lesion. On light microscopy, 500 cells were examined in the basal and parabasal epithelial layers of biopsy samples at 400 \times magnification. All biopsy samples were topo II α positive and approximately 70% of

the counted cells were positive. Strong staining of topo II α was significantly correlated with dysplasia ($P = 0.019$), basal cell hyperplasia ($P = 0.005$) and ulceration ($P = 0.008$) in the samples. Ki-67 was expressed in all samples but only 36% of the cells were positive. CK-19 positivity was found in 29% of the specimens. Histological parameters were not related to either Ki-67 or CK-19. The comparison of the staining patterns with the DNA content of lesions showed that strongly stained cells with topo II α were significantly more frequent in the samples with 2.5cER higher than 15% than in those below 15% ($P = 0.013$; Mann–Whitney). The percentage of the measured cells is 2.5cER exceeding the 2.5c value on the DNA scale. We earlier showed that this cut-off value of 2.5cER discriminated DNA aneuploidy. To conclude, topo II α is a proliferation and also an apoptotic marker in atrophic OLP lesions and it might have a predictive value in oral lichen planus lesions prone to develop malignancy.

Keywords Oral lichen planus · Topoisomerase II α · Ki-67 · CK-19 · DNA content

Abbreviations

OLP	Oral lichen planus
topo II α	Topoisomerase II α
CK-19	Cytokeratin-19
ER	Exceeding rate
PI	Proliferating index

Introduction

Oral lichen planus (OLP) is a chronic mucocutaneous inflammatory disease, which WHO has defined as a

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potentially precancerous condition, representing a generalized state, associated with a significantly increased risk of cancer [32]. Because of its unknown etiology, the management of OLP is still not satisfactory and the optimal treatment remains to be defined [27]. Currently, there are no prognostic markers to identify the lesions at increased risk for malignant transformation among the chronic OLP lesions.

Topoisomerase II alpha (topo II α) is an enzyme that can modify (isomerise) the tertiary structure of DNA without changing its primary structure, determined by the nucleotide sequence. It exerts an important role in DNA topology, repair and replication by breaking and rejoining the DNA double helix [2]. Topo II α is a cell cycle-related protein and is expressed in normal as well as neoplastic cells in the S, G2 and M phases. The lowest level of expression is found in the G0 and G1 phases. Consequently, topo II α is related to cell proliferation [13, 14] but also related to cell viability and apoptosis. Previous studies have shown that aberrant expression of topo II α is associated with the induction of apoptosis [19, 30].

A variety of other markers of cell proliferation are available as well. Ki-67 antigen is a nuclear matrix protein that is expressed in proliferating cells during the G1, S, G2 and M phases of the cell cycle but not in resting cells (G0 phase) [4, 26]. Ki-67 is the most frequently used proliferation marker in routine diagnostics [31]. There are studies suggesting Ki-67 as a useful prognostic factor in oral squamous cell carcinoma [8], although some other studies did not find any association between Ki-67 and oral cancer [11, 24].

Cytokeratin 19 (CK-19) is an intermediated filament protein, which is found in most of the simple and non-keratinizing stratified epithelia [21]. It was shown as a useful marker of cellular atypia, associated with pre-malignant lesions in oral epithelium [16]. In addition a sparse basal expression of CK-19 is usually detected in OLP lesions [30]. CK-19 expression in suprabasal cell layers of oral mucosa might also be a useful marker for diagnosing oral precancerous lesions. CK-19 expression has been regarded important in the initial events during oral carcinogenesis [22].

We have recently described changes in the DNA content in atrophic oral lichen planus lesions measured by static cytometry and found that during the follow-up DNA content might be a useful predictor of OLP lesions prone to malignant transformation [18]. Based on our microarray studies we also found that topo II α , Ki-67 and CK-19 were among the up-regulated genes in oral cancer cell lines derived from oral lichen planus [25]. Thus these markers were tested in atrophic OLP

lesions to estimate their usability in predicting the lesions at risk for malignancy.

Materials and methods

The material of this study consists of 81 biopsy specimens taken from 70 consecutive patients with atrophic OLP defined as criteria by WHO [32], identified between years 1991 and 2002, and retrieved from the files of the Department of Oral Pathology and Radiology, University of Turku. All patients, born before 1928, those with any medication or suffering from any systemic disease, were excluded from this study. Of the 70 patients, 53 (75.7%) were women with the mean age of 53.3 years (range 29–73 years) and 17 (24.3%) were men with the mean age of 52.7 years (range 36–72 years). Thus the patients represented a typical age and sex distribution of OLP patients. The mean follow up time of the patients was 63.5 months (range from 7.2 to 117 months). Five patients with atrophic oral lichen planus developed oral squamous cell carcinoma during the follow-up. At the first visit all lesions were clinically typical atrophic OLP fulfilling the WHO criteria, such as presence of bilateral lesions on oral mucosa with atrophic or ulcerative outlook. All the specimens stained here were the same as were used earlier for DNA content measurements allowing the comparison between immunohistochemistry and DNA data [18].

Histological examination

The biopsies were fixed in formalin and processed to paraffin-embedded tissue blocks according to the routine practice as described earlier. Haematoxylin and eosin stained slides (6 μ m) were cut for routine diagnostics. Re-examination of the slides confirmed the diagnosis of OLP in all cases. The WHO criteria [32] for OLP were used when re-examining the histopathology of the sections. In addition, the following morphological parameters were recorded: keratosis, atrophy, ulceration, hyperplasia and dysplasia. Also the location of OLP lesion in the oral mucosa was recorded.

The presence of dysplasia was graded as mild, moderate or severe. Dysplasia was mild when dysplastic changes were present in lower third of the epithelia, moderate when two thirds of the epithelia were affected and severe when the whole thickness of epithelia was involved. Basal cell hyperplasia was defined as increased density of basal cells within a unit area.

Immunohistochemistry

For immunohistochemical staining of topo II α , Ki-67 and CK-19, 4 μ m thick sections from formalin-fixed, paraffin-embedded tissue blocks were cut. Briefly, the sections were de-paraffinized and boiled in microwave for 2 \times 5 min in 0.01 M citrate buffer pH 6, then after cooling in room temperature for 20 min. The monoclonal antibodies for topo II α , Ki-76 and CK-19 were used (DAKO, Glostrup, Denmark) at a 1:100 dilution, ready-to-use, and 1:100, respectively. The staining was made by DAKO TechMate™ 500 Plus staining system, following the manufacturer's instructions. Sections from breast carcinoma were used as a positive control for topo II α and CK-19, while a biopsy of oral verruca vulgaris was used as a positive control for Ki-67. Negative controls were processed in parallel to test samples, by replacing the primary antibody by buffer.

Evaluation of immunostaining

On light microscopy, 500 cells in each section were examined for the staining of topo II α and Ki-67 in the basal and parabasal epithelial layer at 400 \times magnifications. The staining pattern was graded as negative, diffuse staining (<50%), and strong staining (\geq 50%) comparing to the intensity and frequency of stain among the cells (Figs. 1, 2). The cell was graded as diffuse when the staining of the nucleus was punctuated and as strong when the whole nucleus was in dark staining. Staining pattern of CK-19 was graded into four groups: (1) no staining, (2) only few cells staining, (3) less than 50% of the cells stained, and (4) 50% or more of the basal cells stained (Fig. 3). For immunohistochemical stainings consecutive sections were made

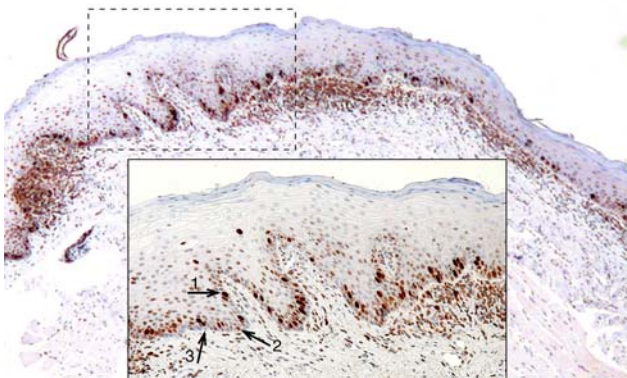


Fig. 1 Immunohistochemical staining of topo II α in an atrophic OLP biopsy sample. *Arrow 1* illustrates the strong staining of the cell, *arrow 2* illustrates diffuse staining of the cell and *arrow 3* illustrates no staining of the cell

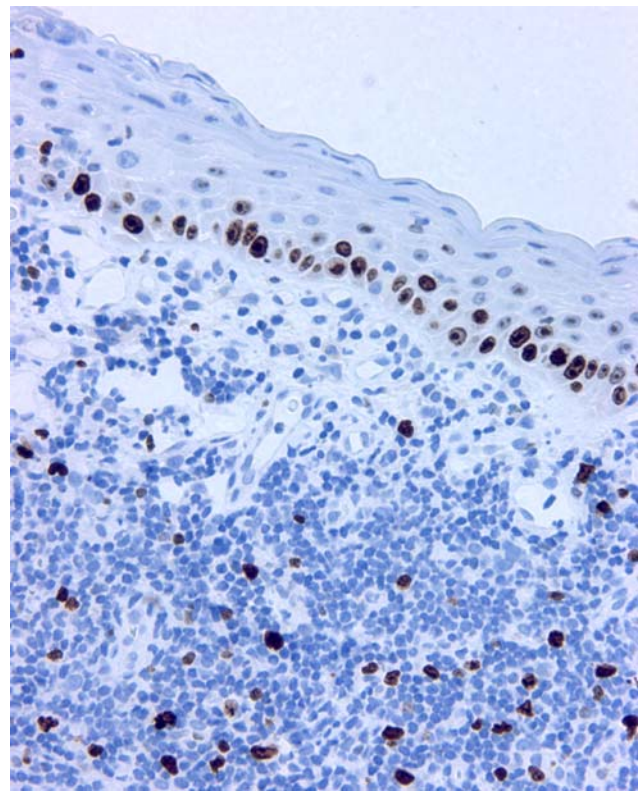


Fig. 2 Immunohistochemical staining of Ki-67 in an atrophic OLP biopsy sample (original magnification \times 200)

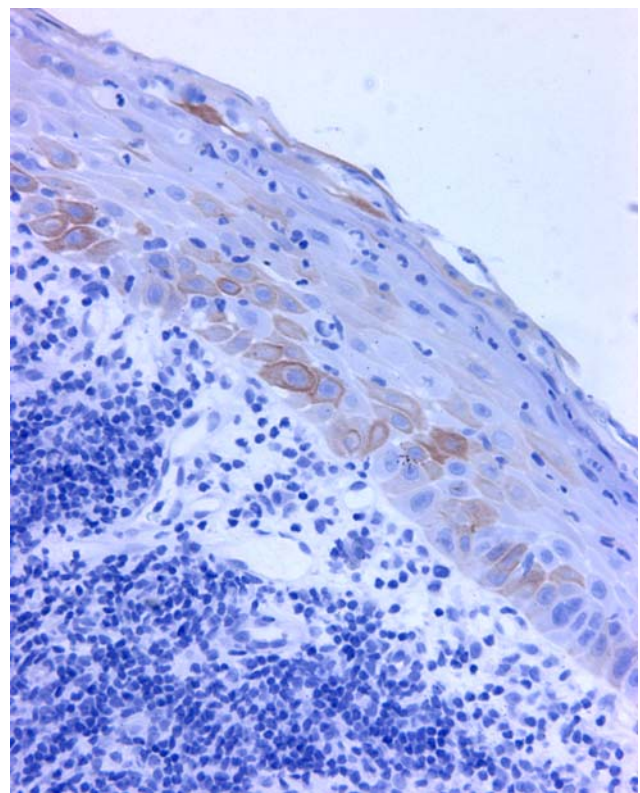


Fig. 3 Immunohistochemical staining of CK-19 of the same sample as presented in Fig. 2 (original magnification \times 200)

from the specimens. Thus, it was possible to correlate the location of expression of topo II α , Ki-67 and CK-19 with each other.

DNA analysis

For analysis of the DNA content, we used static cytometry, as previously described, analyzing a wide variety of parameters [18]. DNA ploidy is 1 (one) in a normal diploid cell and 2 (two) in a dividing or tetraploid cell. In an aneuploid cell the ploidy could be approximately between 1.2 and 1.8. In our study the approximately value for ploidy discriminating DNA content in cells was 1.3. Its importance proved to be 2.5c exceeding rate (2.5cER) and the proliferation indexes (PI). The rate of deviation from the 2.5c value (sign of diploidy with cell in G0 phase) is represented by 5cER, is the percentage of nuclei that are over 5c (polyploidy or aneuploidy). Proliferation index is the sum of S and G2/M phase fractions. Determined on the basis of the previous analysis, the value of 15.3% for 2.5cER was selected here as the cut-off value. The statistically significant value for 2.5cER explaining the presence of DNA aneuploidy was 15.3% in our previous study. All these values recorded in static cytometry were compared with the immunohistological staining for topo II α , Ki-67 and CK-19.

Statistical analysis

SPSS for Windows, version 11.5 (Chicago, IL, USA) was used for statistical analysis. Frequency tables were analyzed using the Chi-square test, with Pearson’s R or likelihood ratio (LR) being used to assess the significance of the correlation between the categorical variables. Differences in the means of continuous variables between the groups were analyzed using ANOVA (when appropriate) or non-parametric tests (Mann–Whitney, Kruskal–Wallis) tests. Binary logistic regression models were used to analyze the power of different variables as predictors of dysplasia, using the stepwise backward approach. In all analyses, the probability values less than 0.05 were regarded as significant.

Results

During the follow-up the oral lichen lesions progressed to squamous cell carcinoma in five patients. Table 1 shows the results of immunohistological stainings in the original OLP lesions of these patients before cancer development. Topo II α was expressed in 75% of

Table 1 The mean percentage of Topo II α , Ki-67 and CK-19 stained cells of the five original biopsy samples taken from patients originally diagnosed with atrophic lichen planus

Age, sex	Location	Ulceration in the original biopsy sample	Dysplastic changes in the original biopsy sample	25cER	5cER	Ploidy	Follow-up	Topo II α		Ki-67		CK-19	
								No staining % ^a	Diffuse staining % ^a	No staining % ^a	Strong staining % ^a	No staining % ^a	Strong staining % ^a
41 years, female	Tongue	No	No	24.0	0	2.0	Cancer in 17 months	13.0	71.2	15.8	56.0	13.0	No staining
54 years, female	Cheek	No	No	13.9	0	2.0	Cancer in 61 months	22.2	61.6	16.4	53.2	19.2	Only few stained cells
58 years, female	Tongue	Yes	No	14.5	0	2.1	Cancer in 7 months	14.0	65.6	20.4	34.2	20.6	No staining
69 years, female	Tongue	No	No	72.3	1.3	2.0	Cancer in 59 months	26.6	64.4	9.0	85.4	3.0	No staining
62 years, male	Tongue	No	Moderate	43.8	3.1	1.9	Cancer in 20 months	86.8	6.8	6.4	81.2	8.4	No staining

The lesions subsequently developed to oral squamous cell carcinoma

^a A total of 500 cells were counted

the counted cells in four of the five biopsy samples. One atrophic OLP progressed to carcinoma in only 7 months. In this lesion 20% and 45% of the cells were strongly stained with topo II α and Ki-67, respectively.

In eight of the 81-biopsy specimen there were dysplastic changes present in the original biopsy samples. During the follow-up (mean 63.5 months) there was no progression of dysplasia in seven of those lesions. Only one of the eight lesions progressed to cancer in 20 months from the original diagnose of atrophic OLP. The rest of the OLP lesions had no changes during the follow-up time.

We compared the expression of topo II α in patients who developed oral SCC and in those who did not. There was a borderline significant difference of topo II α expression between these two groups (Fisher's exact test; $P = 0.054$). There was no such difference in expression of Ki-67 and CK-19.

Table 2 summarizes the staining pattern of topo II α and Ki-67 in OLP. The assessment was based on 500 cells counted in each biopsy sample. In every sample there were cells without any staining, with diffuse staining and strong staining. For topo II α , diffuse staining pattern was detected in 59% of the cells, while 11% of the cells were strongly stained. Diffuse staining for Ki-67 was detected in 12% of the cells, while strong staining was detected in 24% of the cells.

The staining pattern of CK-19 is shown in Table 3. CK-19 expression was detected in 24 of the 81-biopsy samples (29%). Of the positive samples, 11/24 was classified in category <50% of the cells stained while only in four samples 50% or more of the cells were stained.

Histological variables related to immunohistochemical staining patterns

In dysplastic lesions, there were statistically significantly more strongly ($P = 0.019$) (mean 58.7 ± 16.6 SD) and diffusely stained ($P = 0.030$) (mean 11.5 ± 6.3 SD) topo II α positive cells than in non-dysplastic lesions. Strong and diffuse staining of topo II α was also related to basal cell hyperplasia ($P = 0.005$) (mean 14.0 ± 5.4 SD) and ulceration ($P = 0.008$) (mean 48.4 ± 18.9 SD),

respectively. There was no significant relation with topo II α and location of the lesions.

The staining pattern of Ki-67 was not statistically significantly associated with any specific histological parameters or location of the lesions.

The staining of CK-19 was significantly more frequent in the tongue than in other sites of oral mucosa ($P = 0.047$). However, CK-19 had no statistically significant relation to any of those histological parameters mentioned above. Moderate dysplastic changes occurred in one of the strongly stained lesions and one lesion with only few stained cells.

We have earlier determined the DNA content in these biopsy samples. Table 4 summarized the relationship of topo II α expression with the DNA content. There were 34 samples with 2.5cER higher than 15% shown earlier to be useful cut-off value to detect pre-malignant lesions. The proportion of topo II α -stained cells in these lesions was $65.9 (\pm 31.8$ SD). Strongly stained cells with topo II α were significantly more frequent in samples with 2.5cER higher than 15% than in those below 15% ($P = 0.013$; Mann–Whitney). Based in our previous study we have found 1.3 as a useful cut-off value for ploidy. Strongly stained cells with topo II α were significantly more frequent in samples with ploidy higher than 1.3 than those below 1.3 ($P = 0.034$; Mann–Whitney) (Table 4). However, there were only two samples with ploidy higher than 1.3 of the percentage of stained cells was $20.4 (\pm 2.3$ SD). Finally, topo II α was correlated to G2/M parameter. In strong staining topo II α correlated significantly to G2/M fraction ($P = 0.036$).

G2/M fraction was the only DNA parameter, which was significantly associated either with diffuse staining pattern of Ki-67 ($P = 0.046$) or without any staining

Table 2 Immunohistochemical staining pattern for topo II α and Ki-67 in OLP lesions

	Topo II α expression		Ki-67 expression	
	Mean number of the cells/500 N \pm SD	Percentage of stained cells ^a % \pm SD	Mean number of the cells/500 N \pm SD	Percentage of stained cells ^a % \pm SD
No staining	149 \pm 92.8	29.8 \pm 18.1	321 \pm 66.0	64.1 \pm 13.2
Diffuse staining	294 \pm 83.0	58.8 \pm 16.6	62 \pm 32.3	12.4 \pm 6.5
Strong staining	57 \pm 31.3	11.4 \pm 6.3	117 \pm 50.3	23.5 \pm 10.1

^a A total of 500 cells counted

Table 3 CK-19 expression pattern in OLP lesions

CK-19 staining pattern	Number of specimens	%	Total %
No staining	58	70.7	70.7
Only few stained cells	9	11	29.3
<50% of the cells stained	11	13.4	
\geq 50% of the cells stained	4	4.9	

Table 4 Association of topo II α expression with 2.5cER and DNA ploidy

Topo II α Strong		Number of samples	Percentage of stained cells M \pm SD	Level of significance (<i>P</i> -value)*
2.5cER %	<15	48	51.2 \pm 29.8	0.013
	\geq 15	34	65.9 \pm 31.8	
	Total	82	57.3 \pm 31.3	
DNA Ploidy	<1.3	80	11.2 \pm 6.1	0.034
	\geq 1.3	2	20.4 \pm 2.3	
	Total	82	11.5 \pm 6.3	

*Mann–Whitney test

($P = 0.024$). CK-19 had no significant associations with any of the DNA parameters tested.

Mutual correlations showed that topo II α was statistically significantly correlated to strong staining of Ki-67 ($P = 0.000$; Pearson correlation), ($P = 0.002$; Spearman's rho).

Discussion

The aim this study was to investigate the expression of topoisomerase II α , Ki-67 and cytokeratin 19 in atrophic oral lichen planus as a prognostic and proliferation marker. The results were correlated with histological parameters and our recent results with static cytometry [18].

Previous immunohistological studies have shown topo II α to be a reliable indicator of cell proliferation in carcinomas of vulva [6], breast [17], uterine [9] and head and neck [12, 28]. In prostate carcinoma expression of topo II α seems to relate to the grade of the carcinoma being lowest in well-differentiated neoplasms and highest in poorly differentiated tumours [33]. In the present study, the topo II α was expressed in 70.2% of the counted cells. Our results indicate that atrophic lichen planus is a highly proliferative lesion in oral mucosa. Besides proliferation expression of topo II α could be due to DNA repairing role of topo II α . Similar results of high cell proliferation in oral lichen planus lesions have been reported earlier, even though in those studies the used proliferation marker is Ki-67 together with p53 [10, 29].

We also found that the other cell proliferation marker, Ki-67 was much lesser expressed in OLP lesions than topo II α (35.9 vs. 70.2%). However, strong staining of topo II α correlated statistically significantly with Ki-67 ($P = 0.002$). Our results support the earlier view that topo II α might be a better proliferation marker than Ki-67 [12, 17]. Topo II α expression in the S, G2, and M phases gives a better estimate of the number of the actively cycling cells than Ki-67 does, as shown with static cytometry. Difference can also be explained by the repairing or apoptotic role of topo

II α , which Ki-67 does not have. Static cytometry alone is unable to prove cell repairing, thus further studies are needed.

Besides proliferation marker topo II α repairs DNA damages during cell proliferation [2]. Apoptosis seems to be increased in oral lichen planus [3]. In addition, it is shown that topo II α plays a role in apoptosis by inducing apoptotic cell death [1, 19, 30]. Topo II α could be a marker of unsuccessful DNA repair, which finally will result in apoptosis. This would explain the more frequent expression of topo II α than Ki-67 in atrophic OLPs. Our finding that topo II α correlated with the changes in DNA content further supports the need of DNA repairing in these lesions. In atrophic oral lichen apoptosis is increased but the reason for this is totally unknown. It might be that one key event is DNA failure leading to increased expression of repair markers as topo II α , but the repair might failure by depletion of topo II α leading to apoptosis of basal or parabasal cells [1]. It is also shown, that protein kinase C delta (PKC δ), which has an essential role in the genotoxic stress response, activates topo II α alpha to induce apoptotic cell death in response to DNA damage [34]. Previous studies have shown that intense expression of topo II α was also significantly related to response to chemotherapy [28].

Our results of static cytometry showed that strong staining of topo II α correlated with G2/M fraction ($P = 0.036$), while the association with Ki-67 was slightly different. Not only the diffuse staining of Ki-67 correlated statistically significantly with G2/M ($P = 0.046$) but there was also the no colour pattern ($P = 0.024$). It is known that Ki-67 accumulates during S phase and it rapidly disappears from post-mitotic cells [5]. The rapid disappearing and short half-life of Ki-67 during G2/M phase could be one explanation for the negative staining pattern of Ki-67 found in this study.

Ki-67 is widely used as a molecular marker of cellular proliferation that predicts prognosis of many different tumours. Some studies however cannot prove Ki-67 as a useful indicator for cancer development or recurrence [7, 15]. When we compare the expression of Ki-67 with those of static cytometry the results are not fully

concordant. In atrophic lichen planus expression of Ki-67 is of limited value as a proliferation marker.

CK-19 expression was detected in 29% of the OLPs whereas only 4/81 samples were strongly stained. Suprabasal CK-19 staining has been regarded as a marker of premalignant change in oral epithelium [16]. Our results further support the view that CK-19 can be used as marker of cellular atypia in pre-malignant oral lesions [16, 22]. However, it has to be noted that biopsy sample might not always cover the whole histological picture present in a clinically extensive lesion. Sometimes it might be difficult to target the correct biopsy site.

In the present study, 41% of the OPL lesions were aneuploid [18]. We have reported earlier that 2.5cER and PI values were significantly higher in ulcerated OLP lesions than in nonulcerated ones ($P < 0.001$ and $P = 0.012$, respectively). Furthermore, OLPs located in the tongue had significantly higher values of 2.5cER and PI than lesions at other sites ($P < 0.001$ and $P = 0.013$). We also concluded that atrophic OLP lesions with both ulceration and dysplastic changes were at risk of developing an oral cancer. Based on the present results we can add that an atrophic OLP biopsy sample with strongly topo II α staining and high 2.5cER and DNA ploidy values could be a risk lesion to progress to malignancy.

According to the best of our knowledge, there are no previous studies that compare the results of static cytometry to topo II α or CK-19 staining. Ki-67 has earlier been regarded as a useful prognostic marker for prostate and head and neck carcinomas, although conflicting results exists [20, 23].

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