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Prognostic Evaluation of HPV-associated Precancerous and Microinvasive Carcinoma of the Oral Cavity: Combined Use of Nucleolar Organiser Regions (AgNOR) and Proliferating Cell Nuclear Antigen (PCNA)

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Nucleolar Organiser Regions (NORs) and Proliferating Cell Nuclear Antigen (PCNA) were investigated on routine paraffin embedded histologic sections of 30 oral biopsy specimens (six cases of leukoplakia with low-degree of dysplasia, nine cases of leukoplakia with moderate-degree of dysplasia, nine cases of leukoplakia with severe-degree of dysplasia, six cases of squamous microinvasive carcinomas), tested for HPV-DNA by *in situ* hybridisation (ISH). The absolute number of NORs per nucleus and the percentage of nuclear positivity for PCNA were found to be different in each group of pathology, with further diversity due to the presence or absence of HPV-DNA. In the major part of HPV-positive lesions, the AgNOR number and percentage of cells positive for PCNA were found to be generally lower than in corresponding negative forms. Conversely, a few cases of HPV$^+$ lesions showed significantly higher values both of AgNOR and PCNA, if compared to the other cases of HPV$^+$ and HPV$^-$ lesions. These data suggested that high values of AgNOR and PCNA, in moderate and high grade oral dysplasia, could represent an "alarm signal" of a worse prognosis, and then a possible indication for a strict clinical management and/or a stronger treatment of some HPV-associated preneoplastic lesions.

Keywords: AgNOR, PCNA, HPV, oral carcinoma, preneoplastic lesions, ISH, virus

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INTRODUCTION

ORAL SQUAMOUS carcinogenesis is a multistep process with a multifactorial aetiology involving many factors, such as age, sex, race, genetic background, but traditionally attributed to abuse of tobacco and alcohol [1–3].

Nevertheless, during the last few years, evidence has been provided that human papillomavirus (HPV) is involved both in the development of oral carcinoma and of premalignant mucosal lesions [1, 2, 4–21].

The reported prevalences of HPV DNA in precancerous lesions and carcinomas of the oral cavity are very discordant. In fact it has varied from 0% in Zeuss *et al.* [16], Young and Min [17] and De Villiers [22], 78% in Woods *et al.* [23] to 100% in Watts *et al.* [18], Greer *et al.* [15] and Dekmezian *et al.* [8]. In spite of these disparate data, the finding of a higher number of patients with HPV infection in the oral mucosa who also have various degrees of dysplasias, suggests elaborate methods which permit the identification of cases with faster evolution into invasive carcinoma.

For the oral cavity, as well as for the genital mucosa, it was noticed that types 2, 6, 11 are more frequently associated with condylomata acuminata, verruca vulgaris and some mild dysplasias [2, 9, 16, 20, 21, 24–26], whereas a strong correlation exists among HPV 16, 18, 31, 33, 35, highly dysplastic lesions and invasive squamous cell carcinoma.

Viral type is therefore a possible means to detect high risk lesions, and, therefore, studies which prove that dysplastic epithelium of the oral cavity and squamous cell carcinoma contain HPV types 16 and 18 [6, 8, 14, 15, 27, 28] with high oncogenic potential are of great importance.

But since there are no clear criteria for fixing cases in which HPV is associated with lesions with low, moderate or severe dysplasia or microinvasive carcinoma, we studied and analysed some kinetic parameters of cellular cycle activity in leuko-

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plakial lesions with different degrees of dysplasia and in microinvasive carcinomas tested by *in situ* hybridisation for HPV-DNA, in order to better understand the role of viral infection in carcinogenesis of malignant neoplasias in the oral cavity.

The objects of our study were Nucleolar Organiser Regions (NORs) and Proliferating Cell Nuclear Antigen (PCNA), which were tested to establish whether or not these techniques could be used as prognostic parameters for preneoplastic lesions of the oral cavity [29–35].

The AgNOR technique is widely accepted and can be applied to routine sections to study cellular interphase in tumours and preneoplastic lesions for a correlation with microscopic diagnosis and prognosis; simple silver stain is used to show Nucleolar Organiser Regions' (NORs), DNA fragments with ribosomal genes localised on short arms of five pairs of acrocentric chromosomes 13, 14, 15, 21, 22 [36], with an important role in nucleolus formation.

Each of these chromosomes carries two NORs, therefore the NOR number in a normal diploid cell is 20 [30], but NORs are aggregated typically in one or two nucleoli during the nuclear interphase.

NORs codify for ribosomal RNA and can be shown in sections obtained from paraffin embedded tissue by virtue of argyrophilia of associated proteins (RNA polymerase I, C23, B23) [30].

The importance of this technique is due to the fact that all the argyrophil bodies shown in the nuclear interphase are AgNORs and their numbers correlated positively with ribosomal gene activity and, therefore, cellular proliferation. Malignant cells contain more AgNORs compared with benign cells [32, 34, 37–39]; furthermore, high-degree malignant forms often contain more AgNORs than corresponding low-degree forms [31, 40].

NORs, by mild silver stain, appear like black granules which can be counted and are defined as AgNOR [38].

On the other hand, Proliferating Cell Nuclear Antigen (PCNA) is an autoantibody which recognises KD36 acid nuclear protein involved directly in DNA synthesis, reaching an expression peak during the S-phase of the cellular cycle and playing a role in cellular proliferation [33, 34, 41–44]. The gene for human PCNA has recently been cloned; it is transcribed effectively both in quiescent and proliferating cells, but mRNA for PCNA accumulates normally only in proliferating cells. In other words, PCNA immunoreactivity shows the "proliferating compartment" in the context of some forms of neoplasia (on routinely fixed and processed tissues), so providing useful kinetic information.

Only one study was carried out testing PCNA [33] in oral cavity carcinomas and few others on AgNOR [29, 31, 32, 34] in preneoplastic and neoplastic lesions, without considering the possible presence of HPV infections.

So, as previously performed for other pathologies (well-differentiated cutaneous squamous cell carcinoma and keratoacanthoma [45], carcinoma *in situ* of the vagina [46]), we tested AgNORs and PCNA in preneoplastic and neoplastic lesions of the oral cavity associated with HPV infection to establish the utility of these techniques as prognostic markers.

MATERIALS AND METHODS

For this study 30 oral biopsy specimens were used; 15 cases were from men and 15 cases were from women with an average age of 49 years (Table 1).

Table 1. Cases

Cases	Age (years)	Sex	Site
1	42	M	Floor of mouth
2	38	M	Tongue
3	45	F	Floor of mouth
4	26	M	Buccal mucosa
5	51	F	Gingiva
6	61	F	Tongue
7	22	F	Floor of mouth
8	39	M	Palate (hard)
9	43	F	Commissure
10	65	F	Buccal mucosa
11	36	M	Buccal mucosa
12	31	M	Tongue
13	58	F	Commissure
14	72	F	Commissure
15	26	F	Tongue
16	32	M	Buccal mucosa
17	64	M	Floor of mouth
18	39	M	Gingiva
19	49	F	Tongue
20	53	F	Palate (hard)
21	64	M	Commissure
22	51	M	Buccal mucosa
23	47	F	Commissure
24	62	F	Buccal mucosa
25	71	M	Tongue
26	55	F	Floor of mouth
27	49	F	Floor of mouth
28	58	M	Buccal mucosa
29	60	M	Tongue
30	63	M	Gingiva

On these specimens histologic diagnoses of leukoplakia with different degrees of dysplasia and microinvasive squamous carcinoma were carried out and they were distributed as follows:

six cases of leukoplakia with low-degree of dysplasia;
nine cases of leukoplakia with moderate-degree of dysplasia;
nine cases of leukoplakia with severe-degree of dysplasia (carcinoma *in situ*);
six cases of squamous microinvasive carcinoma.

For each case sections (3 μm) of routinely processed specimens were dewaxed in xylene and rehydrated through ethanols to deionised distilled water; then *in situ* hybridisation (ISH) for the HPV-DNA, AgNOR technique and an indirect immunoperoxidase technique with the monoclonal antibody PCNA (PC10, Dakopatts) were respectively performed.

The ISH was carried out by using biotinilated DNA probes provided with a commercial kit (Vira Type "*in situ*", Life Technologies, Gaithersburg, Maryland, U.S.A.), for the detection of HPV types 6/11, 16/18, 31/33/35, in routinely processed archivial biopsies. Tissue processing, pretreatment, hybridisation and detection were carried out according to the manufacturer's instructions with some modifications.

Briefly, the sections were mounted on multiwell slides (Hendley, U.K.), coated with 2% aminopropyl-ethoxylane in acetone, dewaxed and target (viral DNA) was unmasked by digestion with the protease/hydrochloric acid solution provided with the kit.

After washing in phosphate buffered saline (PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, pH 7.4) and air

drying, sections were covered with 6–20 µl of hybridisation mix (HM) containing a cocktail of biotinilated HPV-DNA probes (Life Technologies, Gaithersburg, Maryland, U.S.A.); each one was singly probed with combined HPV 6/11, HPV 16/18 and HPV 31, 33, 35, respectively.

Sections and probes in HM were covered by glass coverslips, denatured simultaneously at 100°C for 5 min and hybridised at 37°C for 2 h. After hybridisation, samples were washed twice at 37°C in 0.1 × SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate) containing 50% formamide and soaked in blocking agent (TBT) containing 3% bovine serum albumin, 0.05% Triton X100 in TBS (50 mM Tris–HCl, 100 mM NaCl and 1 mM $MgCl_2$, pH 7.2).

Samples were incubated in streptavidin–alkaline phosphatase conjugated (Life Technologies, Gaithersburg, Maryland, U.S.A.) for 20 min at 37°C in a humidified chamber. After a thorough washing in TBS, sections were incubated in nitroblue tetrazolium chloride (NBT)-5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) substrate in TBS buffer for 1 h at 37°C. The reaction was terminated by washing in distilled water and sections were then counterstained with nuclear Fast Red for 1 min [47].

The AgNOR technique was performed according to Ploton *et al.* [40]. Briefly, the silver colloid solution for staining NORs was prepared by dissolving gelatine in 1% aqueous formic acid at a concentration of 2%. This solution was mixed, 1:2 volume, with 60% aqueous silver nitrate to obtain the final working solution.

This was dropped on the sections and then left for 60 min at room temperature, protected from daylight; sections were then washed with deionised water and counterstained with Mayer's haematoxylin.

The sections were taken to xylene and mounted in a synthetic medium. The usual controls were performed as described by Crocker and Nar [39].

Visible dots in 100 cells per section were counted blindly, at 1060 × magnification under oil immersion, independently by two observers. Hence, the mean score for each case and for each observer, was calculated. Finally, a statistical analysis on each group of lesions and between all the groups was performed using a two-sided *t*-test (Student's *t*-test) for independent samples.

PCNA staining was performed using a conventional indirect immunoperoxidase technique on routinely fixed tissues.

The activity of endogenous peroxidases was blocked by methanol containing H_2O_2 at 3% for 30 min. Sections were treated with normal bovine serum diluted at 1% in PBS for 30 min to reduce the non-specific reactions. Sections were then incubated for 1 h at room temperature with PCNA monoclonal antibody (PC10, Dakopatts) diluted 1:50; the conventional biotin–streptavidin method was performed. The reactivity was evidenced with diaminobenzidine and H_2O_2 at 0.03%. Sections were counterstained with haematoxylin.

The final stain is almost entirely confined to the nucleus and may show a diffuse or granular pattern, or a mixture of both.

Mitotic cells usually fail to show any positivity for PCNA, whereas some of these show an artefactual diffuse staining throughout the cell, because the nuclear membrane is lost during mitosis.

In this, as in previous studies [48], all nuclear staining was considered as positive, regardless of its nature.

Random fields were sampled with the aid of a random table, and 1000 cells were counted using a ×40 objective. The PCNA index was calculated by comparing the number of cells with positive staining with the total number of cells counted, expressed as a percentage.

This was found to be accurately reproducible, with errors of less than ±3%.

RESULTS

HPV-DNA was found by ISH in 17 of 30 cases; the viral types involved were HPV 6/11, 16/18 and 31/33/35. These results, related to the AgNORs and PCNA values, are reported in Table 2. The data concerning AgNORs are further analysed in Table 3, where the numeric differences in each group and among all the groups of lesions were calculated by using a two-sided *t*-test for independent samples to compare the means. Furthermore, a *k*-test was performed to verify the inter-observer reproducibility. The percentage of cells with a nuclear positivity for the PCNA monoclonal antibody (calculated at 40 ×) ranged from 20 to 30% in leukoplakias with low dysplasia, from 20 to 40% in leukoplakia with moderate dysplasia, from 20 to 90% in leukoplakias with severe dysplasia (ca *in situ*) and from 50 to 90% in microinvasive carcinoma (Table 2).

OBSERVATIONS

Even if there is a significant difference in AgNOR number between low- and high-degree dysplasias [34] (Figs 1–4) this technique is of scarce practical use for a subtler distinction between low and moderate dysplasias due to the existence of a frequent superimposition of values (Table 2). Moreover, in leukoplakias with low degree of dysplasia there was no difference in AgNOR count between HPV-positive (HPV^+) and HPV-negative (HPV^-) lesions (low-degree of dysplasia in HPV^+, average 4.547, DS 0.055; in HPV^-, average 4.250, DS 0.312) (Figs 1, 3). In moderate dysplasias the AgNOR number was also almost the same in HPV-positive and negative lesions (HPV^+, average 5.276, DS 0.436; HPV^-, average 5.653, DS 0.050) (Table 2).

Values concerning PCNA showed some differences between HPV^+ and HPV^- lesions: in fact they were 25–30% in HPV^- lesions with low/moderate dysplasia and 20–25% in all (Figs 1, 3) (except two) HPV^+ lesions, whereas there was a peak positiveness of 38–40% in two cases of moderate dysplasia, both of them positive for HPV 6/11 (Table 2).

Data concerning severe dysplasias, where in almost all cases it was possible to distinguish HPV^+ and HPV^- lesions (Figs 2, 4), are also very interesting. In fact, whereas in HPV^- severe dysplasias AgNORs' average was 6.900, DS 0.046 (Table 2), and the positiveness for PCNA ranged from 35 to 90% (Table 2, Fig. 4) (the case with more AgNORs per nucleus also had a higher positiveness for PCNA), in HPV^+ severe dysplastic lesions AgNORs' average was considerably lower (average 6.283, DS 0.520 except two cases with an average of 7.00 and 6.90, respectively) (Table 2, Fig. 2) and immunopositiveness for PCNA ranged from 20 to 25% (Table 2, Fig. 2).

The two cases of HPV^+ severe dysplasia (HPV 16/18 and 6/11) with a higher AgNOR number (with an average of 6.90 and 7.00 per nucleus, respectively) had a positiveness for PCNA of 40 and 70% (Table 2).

Considering data concerning HPV microinvasive carcinomas, AgNORs' average was 7.950 and standard deviation was

Table 2. AgNOR values and PCNA immunopositivity in dysplasic lesions of oral mucosa

Case	HPV*	AgNOR (Average HPV⁺/HPV⁻, DS)			PCNA† (%)
Leukoplakia					
Low-degree of dysplasia		Average‡	Average§	DS	
1	16/18	4.55			20
2	16/18	4.49	4.547	0.055	20
3	6/11	4.60			25
4	Negative	4.00			30
5	Negative	4.15	4.250	0.312	28
6	Negative	4.60			25
Moderate-degree of dysplasia		Average‡	Average§	DS	
7	16/18	5.70			20
8	6/11	5.80			40
9	6/11	5.00	5.276	0.436	38
10	6/11	4.99			20
11	16/18	4.89			22
12	Negative	5.60			30
13	Negative	5.70			28
14	Negative	5.69	5.653	0.050	26
15	Negative	5.62			25
Severe-degree of dysplasia		Average‡	Average§	DS	
16	16/18	6.90			40
17	6/11	7.00			70
18	16/18	5.89			20
19	16/18	5.90	6.283	0.520	25
20	6/11	6.00			20
21	31/33/35	6.01			20
22	Negative	6.89			40
23	Negative	6.89	6.900	0.046	35
24	Negative	6.92			90
Microinvasive carcinoma					
		Average‡	Average§	DS	
25	16/18	7.88			60
26	6/11	7.80	7.830	0.044	50
27	16/18	7.81			90
28	Negative	7.90			50
29	Negative	7.95	7.950	0.050	55
30	Negative	8.00			75

*Type of HPV (*in situ* hybridisation).
†% of positive cells for PCNA (40×).
‡AgNORs' average between observers.
§AgNORs' average between HPV⁺ or HPV⁻ cases.

0.050, with a positiveness for PCNA ranging from 50 to 75% whereas in HPV⁺ microinvasive lesions AgNORs' average per nucleus was 7.830, DS 0.044, with a positiveness for PCNA varying from 50 to 60% (Fig. 5); one case, only HPV⁻ 16/18, with an AgNORs' average of 7.81, showed an extraordinary positiveness of 90% (Table 2).

Data concerning the *t*-test between HPV⁺ and HPV⁻ cases, in each group and in different groups, are reported in Table 3.

DISCUSSION

A previously reported study [34] on oral cavity lesions not associated with HPV infection showed an average of AgNOR values of 4.51 in benign keratoses, 5.61 in not well-differentiated dysplasias, 8.37 in squamous cell carcinomas. In 1991 Sano *et al.* found that in first-degree squamous cell carcinomas of the oral cavity the AgNOR average was 6.39 whereas in second-degree carcinomas the average was 9.74; an association of these lesions with HPV infection had never been reported [31].

The methods to weigh AgNORs include counting AgNOR granules [38, 39, 49, 50] and the determination of area, volume, pattern and distribution according to morphometric criteria [34].

We counted AgNOR granules because it is considered to be a basic method and the obtained values can be superimposed to those reported in the literature [33, 34]. Moreover, in our study was expected to find higher kinetic values in HPV-associated lesions of oral mucosa than in lesions free from viruses with the same histologic degree of dysplasia. On the contrary, some interesting observations can be made considering our final results: one of these concerns the fact that in most cases and without a clear correlation with a specific viral type, the HPV-associated dysplasia seems to have about the same or even lower nuclear activity and proliferating cell index (calculated by combined evaluation of AgNOR and PCNA)

Table 3. Statistical comparison of AgNORs' averages (two-sided-t-test)

(a) Statistical comparison between HPV^+ and HPV cases for either class of lesions of oral mucosa.

	t	*P*
Low dysplasia		
HPV^+/HPV^-	9.330	>0.02
HPV^-/HPV^+	−1.646	>0.05
Moderate dysplasia		
HPV^+/HPV^-	−1.930	>0.05
HPV^-/HPV^+	15.085	<0.001
Severe dysplasia		
HPV^+/HPV^-	−3.048	>0.05
HPV^-/HPV^+	24.442	>0.001
Microinvasive carcinoma		
HPV^+/HPV^-	−4.768	>0.05
HPV^-/HPV^+	4.157	>0.02

(b) Statistical comparison between different classes of lesions of oral mucosa without any correlation with HPV^+.

	t	*P*
Moderate/low	8.517	<0.001
Severe/low	12.041	<0.001
Microinvasive ca/low	109.687	<0.001
Severe/moderate	6.051	<0.001
Microinvasive ca/moderate	76.860	<0.001
Microinvasive ca/severe	43.700	<0.001

than corresponding HPV-negative forms (Tables 2 and 3a). We also previously found these aspects in two studies in which the same techniques were performed on HPV-positive and HPV-negative lesions localised in other areas (personal communications) [45, 46]. These evaluations could suggest the idea that in most cases HPV infection does not increase cellular proliferation. These data are in agreement with others concerning the uterine cervix and studies on keratinocytes of

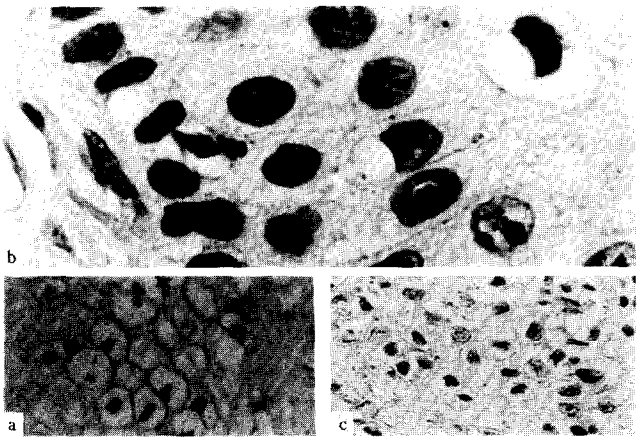

Fig. 1. HPV-positive light dysplasia. (a) HPV 6/11 detected by ISH; (b) AgNOR staining, 1060×; (c) PCNA immunostaining, 25% of positive cells.

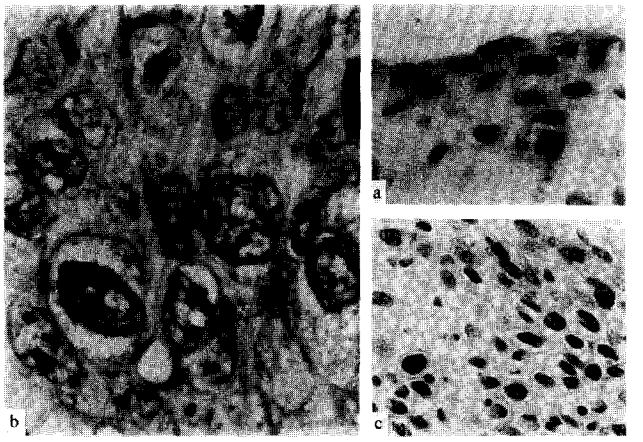

Fig. 2. HPV-positive severe dysplasia (ca *in situ*). (a) HPV 16/18 detected by ISH; (b) AgNOR staining, 1060×; (c) PCNA immunostaining, 70% of positive cells.

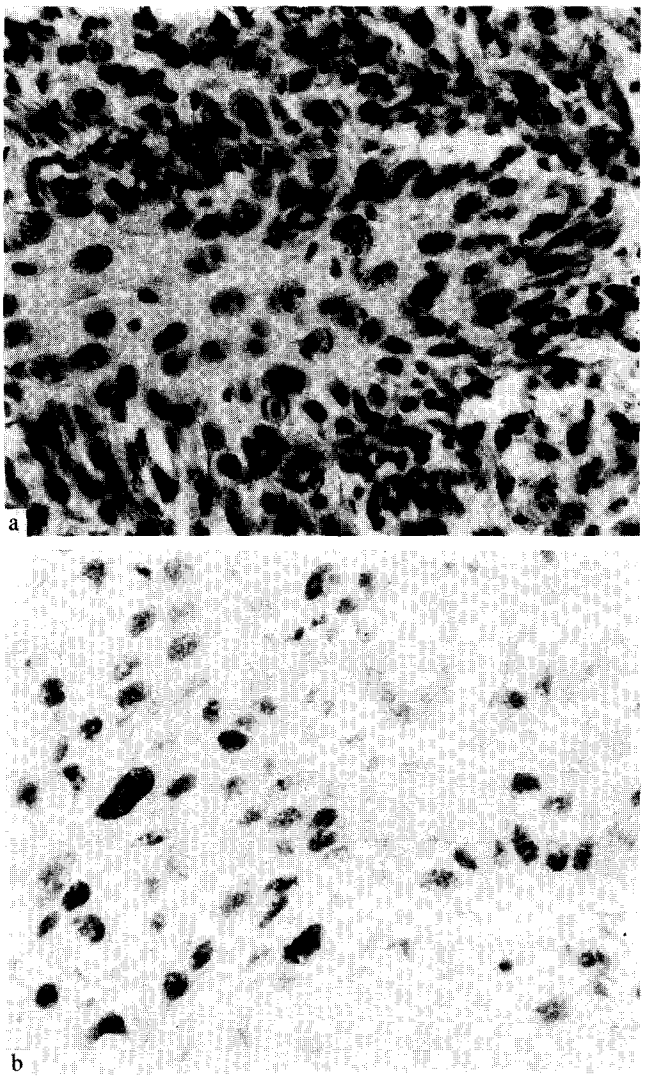

Fig. 3. HPV-negative light dysplasia. (a) AgNOR staining, 1060× (there is a clear partial overlap with Fig. 1(b); (b) PCNA immunostaining: 25% of positive cells.

HPV-infected lesions, showing that the correlation of PCNA positivity with the viral replication was truly uncommon in malignant and most premalignant lesions [26, 51, 52]. Conversely, in condiloma acuminata and low-grade intraepithelial neoplasias there was a consistent induction of PCNA compared with the normal squamous epithelium in which only some of the parabasal and basal cells were positive [52].

There was also a correlation between AgNOR mean number and the progression of cervical lesions with and without HPV infection [53]. Particularly it emerged that there was a different pattern of expression between HPV-negative dysplastic lesions (small and loosely arranged AgNORs) and HPV-positive non-dysplastic lesions (large, compact or single AgNORs) [53].

Therefore, the high frequency of HPV-positive patients in an asymptomatic population could be understood.

Now it must be established why in two of our cases of moderate dysplasia and in two HPV^+ severe dysplasias the AgNOR number and PCNA positiveness were significantly higher compared to corresponding HPV^- forms, without considering the degree of histologic dysplasia.

It is possible to suppose that there are two different patterns of cellular proliferation in intraepithelial HPV-positive lesions beyond the involved viral genotype. Many cases, in fact, had a "light" pattern of cellular proliferation, often equivalent to or even lower than corresponding lesions not associated with the virus.

Only a few cases of HPV-positive patients showed surprisingly higher values of cellular proliferation compared to other cases with or without viral infection, specifically in two moderate dysplasias where values were closer to those found in microinvasive squamous cell carcinomas in which the cancerous process is fully developed.

Taking these aspects into consideration, the AgNOR's high values and also a higher PCNA immunoreactivity in severe and even moderate dysplasias, could represent an "alarm signal" of proliferating cell hyperactivity and, therefore, they might be a possible indication for a stricter clinical management and/or a more incisive treatment of preneoplastic lesions.

But further studies which permit better understanding of the meaning of HPV integration in DNA of oral mucosa cells and its possible relation with the oncogenes' activation and/or repression of tumour suppressor genes are necessary.

Furthermore, a morphometric study of AgNORs in the same pathologies is actually in progress.

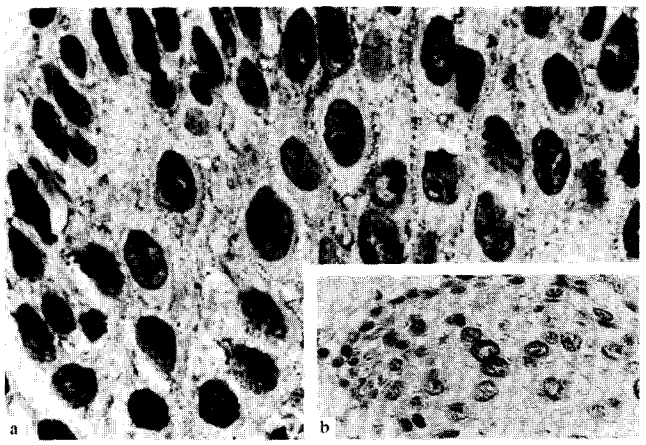

Fig. 4. HPV-positive severe dysplasia. (a) AgNOR staining, 1060 ×; (b) PCNA immunostaining, 40% of positive cells.

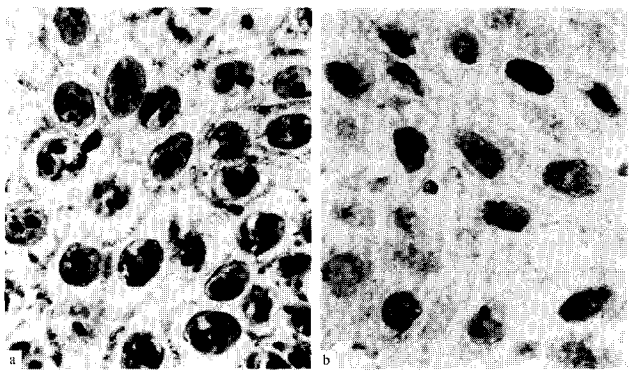

Fig. 5. HPV-positive microinvasive carcinoma. (a) AgNOR staining, 1060 ×; (b) PCNA immunostaining, 50% of positive cells.

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