Toppan Best-set Premedia Limited

Journal Code: JOG	Proofreader: Elsie
Article No: JOG12380	Delivery date: 20 Jan 2014
Page Extent: 8	

doi:10.1111/jog.12380 *J. Obstet. Gynaecol. Res. 2014*

Argentophilic nucleolus organizer region as a proliferation marker in cervical intraepithelial neoplasia grade 1 of the uterine cervix

Fernando Guerra[1], Adriana Esther Rocher[1], José Villacorta Hidalgo[2], Lilí Díaz[2], Susana Vighi[2], Lucía Cardinal[2], Silvio Tatti[3], Nicasio Cúneo[4], Gonzalo Prat Gay[5], Gabriela Camporeale[5] and Luis Alberto Palaoro[1]

[1]Department of Clinical Biochemistry, [2]Department of Pathology, [3]Gynecological Pathology Division, Clinical Hospital (UBA), [4]Marie Curie Oncological Hospital and [5]Leloir Institute Foundation – CONICET, Buenos Aires, Argentina

Abstract

Aim: p16INK4a and argentophilic nucleolus organizer region (AgNOR) can be used as markers for progression of cervical intraepithelial neoplasia grade 1 (CIN1) of the uterine cervix. Our objective was to study the predictive value of the AgNOR technique as a progression marker of CIN1 and its correlation with p16INK4A.

Material and Methods: One uterine cervix biopsy from each of 75 patients with diagnosis of CIN1 was selected. All of these patients underwent a second biopsy, and these were also used for the study.

Results: The second biopsies showed: regression (20 patients), persistent CIN1 (38 patients), progression to CIN2 (10 patients) and progression to CIN3 (seven patients). p16INK4A showed reactivity in 67 of the 75 first CIN1 biopsies: 12 of the 20 cases that cleared the lesions and the 55 cases with persistent or progressive lesions were positive for p16INK4a (specificity: 40%; sensitivity: 100%; positive predictive value [PPV]: 82%; negative predictive value [NPV]: 100%). Samples with AgNOR areas less than 3.0 μ^2 returned in all cases, but patients whose lesions persisted or progressed to CIN2/CIN3, showed AgNOR areas greater than 3.0 μ^2 in 50/55 cases (specificity: 100%; sensitivity: 91%; PPV: 100%; NPV: 80%).

Conclusions: p16INK4a is expressed in a high percentage of returning lesions. AgNOR might be a better marker of proliferation of CIN1 than p16INK4a (PPV = 100%), which means that a value greater than 3.0 μ^2 indicates the persistence or progression of the lesion. As its NPV is 80%, a value of AgNOR area less than 3.0 μ^2 in CIN1 leaves a margin of doubt about the future behavior of the lesion.

Key words: argyrophilic nucleolar organizer region, cervical intraepithelial neoplasia grade 1, p16INK4a, progression of cervical lesions, uterine cervix.

Introduction

Cervical intraepithelial neoplasia grade 1 (CIN1) is commonly associated with the human papillomavirus (HPV) genotypes of low risk (HPV number 6 and 11), so called because these lesions tend to resolve spontaneously, due to the action of the immune system.[1] Nevertheless in some cases, the high-risk types are detected in these pathologies.[2] It is presumed that 12–14% of CIN1 progress to CIN2/CIN3[3] and less than 5% of those lesions, without treatment, will progress to invasive cancer, a process that takes approximately 10–12 years.[4]

Received: September 21 2013.
Accepted: December 13 2013.
Reprint request to: Professor Luis Alberto Palaoro, Avda Forest 1318 4° B –(1427) C.A.B.A, Buenos Aires 1120, Argentina.
Email: luispalaoro@yahoo.com.ar

One of the biggest problems in hospitals is the difficulty in controlling and monitoring those patients who receive treatment for premalignant lesions. Depending of the kind of lesion and the treatment done, the follow-up is from 12 to 24 months. The identification of markers that allow us to predict the progression risk in these early lesions would therefore be a very useful tool for the monitoring and management of those cases.

It has been established that the persistence of the infection with some of the oncogenic genotypes or high-risk HPV is a necessary but not sufficient condition for the development of cervical cancer.[5,6] When there is an infection by high-risk HPV genotypes, the expression of the viral oncoproteins, such as E6 and E7, is more stable, and will stimulate the mitosis of the infected cells.[7,8] The protein E7 will bind to the host protein pRb, which is normally a downregulator of the cell cycle, because in its unphosphorylated mode it binds the transcription factor E2F.[9]

The action of E7 over the cell cycle regulation is reinforced with the activity of the protein E6, which binds to p53, a host factor in charge of the proapoptotic control. Attached to E6, p53 now has a new fate, the proteosomic degradation, mediated by ubiquitination,[10] so the affected cell avoids following the programmed apoptosis pathway.

The viral typification for the identification of oncogenic types of HPV did not show a real positive predictive value (PPV) in the evolution of the low-grade intraepithelial lesions, and also it is a high-cost method.[11]

It is thought that 12–14% of CIN1 will progress to CIN2/CIN3[3] and an even lower proportion of those lesions (without treatment) will progress to invasive cancer;[1] however, 15–30% of Papanicolaou tests that result in diagnoses of low-grade squamous intraepithelial lesion actually represent a CIN2/CIN3.

For this reason, other parameters have been proposed to provide a useful positive predictive marker. One of them is the protein p16INK4a, which accumulates in the infected cells by HPV 16/18 when the oncoprotein E7 is expressed, and is a dependable progress index.[12,13]

Physiologically the role of p16 is to block the activity of the CDK4/6. The protein E7 interrupts the binding of the retinoblastoma protein (pRB) with the transcription factor E2F working as a molecular switch, normally activated by CDK 4/6, and in this way, promotes the cell cycle entry (G1 to S phase) of the infected cells. However, as in this case, E2F is not released by the action of CKD 4/6, but for the protein E7, p16INK4a has no effect in the activation of the cell cycle. The consequence is that p16INK4a accumulates in the nucleus and cytoplasm of the affected cells and can be detected by immunohistochemistry,[14] allowing us to infer progression behaviors from CIN1, independent of the HPV typification.

Argentophilic nucleolus organizer region (AgNOR) is a useful marker in many non-gynecological pathologies.[15] NOR are DNA segments that codify the RNA of ribosomal proteins[16] and normally are associated with other specific nucleolar acid proteins that can be visualized by techniques using impregnation with silver salts.[17] These methods were described initially in the 1980s.[18] Through further analysis, the proteins of these regions were identified during interphase (nucleolin and protein B23), and also in mitosis (RNA polymerase I subunits and transcription factor UBF).[19] In the course of the interphase they are localized in the nucleolus being the center of the ribosomal biogenesis; in this way, the size of the nucleolus has a direct correlation with the ribosome synthesis in proliferating cells and, therefore, in the cell metabolic activity.

AgNOR express the replication capacity of a cell or epithelium, as it correlates with ribosomal synthesis and, therefore, proteins synthesis. The number of particles or the surface occupied in the nucleus is related to the mitosis velocity in many tissues, including the uterine cervix.[15,20–22]

When a CIN1 is diagnosed by cytology and by colposcopy, the gynecologist confirms this with a biopsy. Then, he/she makes a follow-up of the patient, taking new biopsies every 6 months for a period of 2 years. If in that period the lesion persists, he/she performs a local treatment; if the lesion progresses, a more aggressive treatment is done.[23] A complementary assay to cytology and colposcopy performed also in the first biopsy could avoid the next surgical procedure in the follow-up. There are few papers reporting the AgNOR technique in the uterine cervix. In this work, we study the potential predictive value of the AgNOR technique as a progression marker of CIN1, which has an uncertain behavior, and its correlation with the already known marker p16INK4A.

Method

Samples

We selected 90 biopsies of uterine cervix of patients with diagnosis of CIN1 from the Service of Gynecologic Pathology Division of the Clinical Hospital 'José

de San Martín,' Buenos Aires, Argentina. All these patients had a second biopsy taken, on average, 6 months after the first ones, and they were also used for this study. The biopsies were fixed in 5% formaldehyde, embedded in paraffin, sectioned at 2–4 µm and transferred to slides with adhesive (Superfrost Plus-ESCO-Thermo Scientific). After deparaffinizing with xylene, the slides were hydrated and divided into three groups: (i) hematoxylin-eosin (HE) stained; (ii) AgNOR assayed; and (iii) immunohistochemistry for p16INK4a.

From the total of 90 collected biopsies, we selected samples from 75 patients; the other 15 samples were discarded either because they did not provide enough material, the second biopsy was not available, or the period in which the biopsies were taken was longer than 1 year.

All of them presented a first CIN1 diagnosis, and of the second biopsies, 20 had a regression (A), 38 had a persistent CIN1 (B), 10 had a CIN2 and seven had a CIN3 (C).

All biopsies were reviewed by three pathologists, and a sample of Pap (exo- and endocervical) along with a colposcopic examination were performed simultaneously. There was very good correlation between these tests.

AgNOR assay

The smears were incubated in the dark for 25 min with a mixture of silver nitrate 50% (W/V) and (2:1) 1% gelatin (W/V) : 1% formic acid (V/V). After washing with deionized water and 1% sodium thiosulfate (W/V), the slides were dehydrated and mounted with Canada balsam. The average AgNOR areas were determined by image cytometry (Image Pro-Plus, Version 4–5) using the oil immersion objective and selecting 100 cells in each smear, only considering the parabasal layers.[21] Normal squamous stratified epithelium was used to measure the average areas of AgNOR to establish the cut-off. The deep zone of the epithelium was chosen because there the difference between CIN1 that progress or regress might be at a maximum.

The positive controls were biopsies of pre-invasive and invasive lesions of uterine cervix. Biopsies without any HPV lesion were used to obtain the normal media value.

The measurement was made in the nuclei towards the parabasal layers, choosing those that present a bigger proliferation and alteration in an HPV infection. The results were expressed as the average of NOR dots per cell per sample.

Immunoassay for p16INK4a

The citrate buffer antigen retrieval protocol was used to unmask the epitopes of p16INK4a.[24] The slides were immersed in citrate buffer (10 mM sodium citrate in 0.05% Tween 20, pH 6.0) and incubated for 20–40 min at 96–98°C. The slides were cooled in the same buffer for 20 min, and then placed in phosphate-buffered saline (PBS). The sections were incubated with primary antibody at appropriate dilution for 1 h at room temperature. Three monoclonal antibodies were used: Santa Cruz (1/150), DAKO (1/50) and an antibody developed in the Leloir Institute (Buenos Aires, Argentina) (1/200). The three antibodies were applied to every biopsy, except in two cases where the DAKO (antibody) was not used. The agreement in the results was confirmed in control experiments. After washing with PBS, the slides were incubated with biotinylated-secondary antibody for 20 min, rinsed with PBS, covered with streptavidin-peroxidase, rinsed again, and finally incubated with H_2O_2 and dyaminobencidine.[25]

Control samples

Negative controls consisted of samples of normal glandular and stromal cervical tissues. Positive controls consisted of samples of CIN2/CIN3, adenocarcinomas and squamous carcinoma of uterine cervix. The immunoassay negative control consisted of performing the assay after replacing the primary antibody with PBS buffer.

Statistical analysis

Statistical analysis was performed with SPSS 16.0. Data are presented as mean ± standard deviation (SD). A P-value < 0.05 was considered statistically significant. Fisher's exact test was used to test categorical variables. For statistical comparison of independent groups, the Mann–Whitney U-test was used.

Ethics statement

All tissue samples were collected for histologic examination and diagnostic purposes and were thoroughly anonymized for use in this study. Thus no informed consent was needed. This study was approved by the Institutional Review Board at the Clinical Hospital-University of Buenos Aires.

Results

p16INK4a immunohistochemistry

The p16INK4a immunohistochemistry showed reactivity in 65 of the 75 first CIN1 biopsies. The evaluation

criteria to consider a positive reaction were: intensity of the color, diffuse staining in the basal 2/3 of the epithelium, or the whole stratum; it could also be nuclear, cytoplasmic or both.

The expression of p16INK4a was scored as 0 (negative), 1 (focal and weak cytoplasmic staining with or without nuclear staining in superficial or intermediate layers), and 2 (diffuse and strong nuclear or cytoplasmic staining in lower 2/3 or in the whole layers).

From 20 biopsies that cleared the lesion (that had a second biopsy without any viral lesion), eight were negative for p16INK4a reaction, and 12 were positive, with scores of 1 (25%) or 2 (75%). All the lesions that persisted or progressed were positive for p16INK4a (score 1: 17%; score 2: 83%). The scores of the biopsies that progressed to high-grade squamous intraepithelial lesion (HSIL) were 2 in all the cases (Fig. 1) (specificity: 40%; sensitivity: 100%; PPV: 82%; negative predictive value [NPV]: 100%).

All the metaplasic or non-dysplastic atypical epithelia were negative for p16. The CIN2/CIN3 had a very intense staining occupying the whole thickness of the epithelium.

The squamous carcinomas, microcarcinomas and the adenocarcinomas of the uterine cervix showed an intense staining for p16INK4a of all the neoplastic cells (Score 2) (Fig. 2).

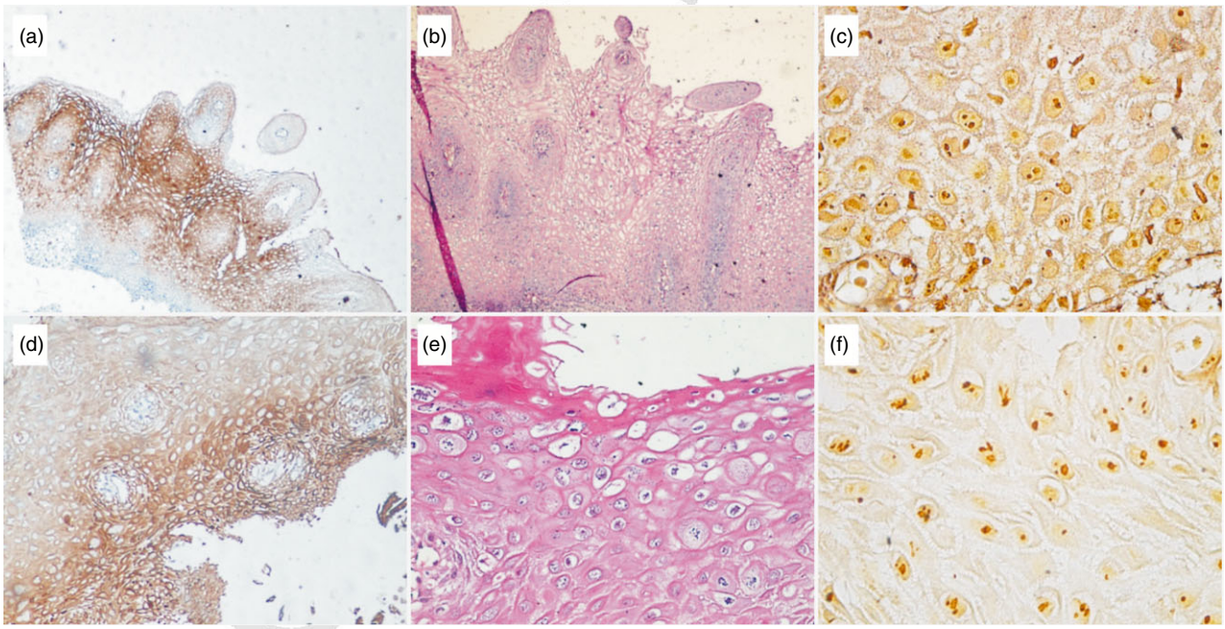
AgNOR

The samples were distributed into two groups: those that revert (Group 1: 20 biopsies) and those that persist as CIN1 or progress to CIN2/CIN3 (Group 2: 55 samples) (Table 1).

The average AgNOR values were: 2.45 μm^2 (SD 0.49) for Group 1 and 3.93 μm^2 (SD 0.72) for Group 2 ($P < 0.0001$) (Table 2). The average AgNOR value of the normal epithelium was $1.81 \pm 1.10\ \mu m^2$ (specificity: 100%; sensitivity: 91%; PPV: 100%; NPV: 80%).

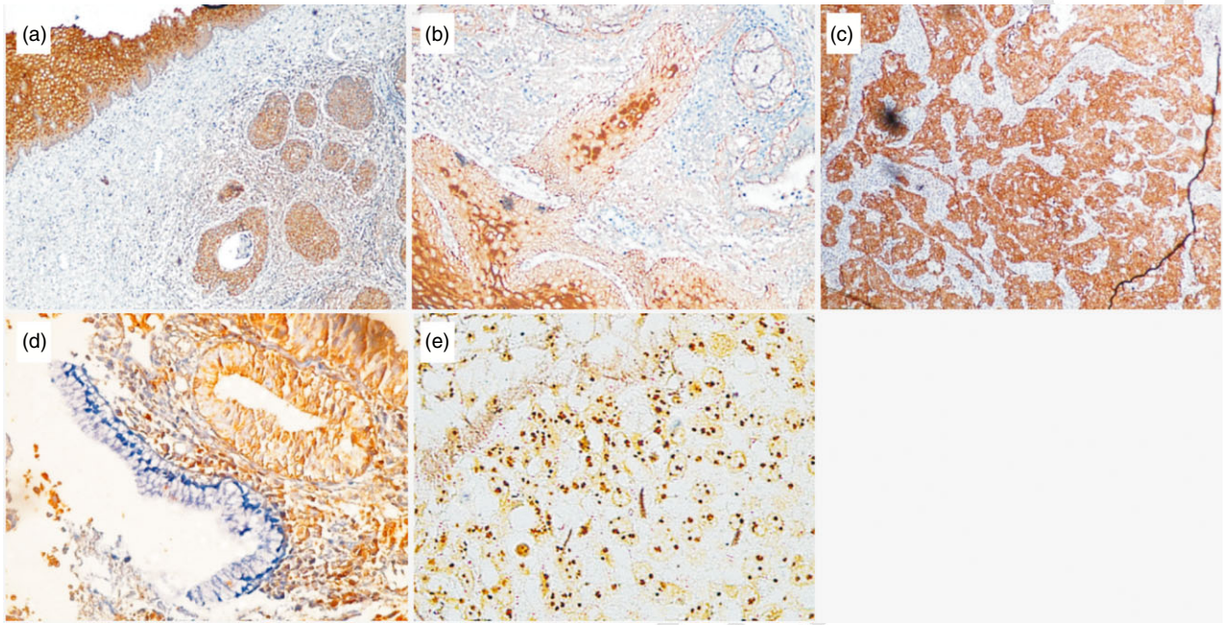
Discussion

The integration of high-risk HPV (HR-HPV) types into the host genome is a necessary step for malignancy, because it leads to increased expression of viral oncogenes (E6/E7). The HR-HPV oncoprotein (E7) inactivates the protein pRb, a necessary step to maintain the



Colour

Figure 1 (a–c) Cervical intraepithelial neoplasia grade 1 (CIN1) that persists: (a) p16INK4a is expressed in the entire thickness of the epithelium. (b) Hematoxylin–eosin (HE). (c) Argentophilic nucleolus organizer region (AgNOR) 4.51 μm^2. (a,b) ×100; (c) ×400. (d–f) CIN1 that progresses to a CIN2. (d) p16INK4a is expressed in the entire thickness of the epithelium. (e) HE. (f) AgNOR 4.13 μm^2. (d) ×100; (e,f) ×400.



Colour

Figure 2 (a) Microcarcinoma of uterine cervix showing strong p16INK4a expression of the neoplastic cells (×100). (b) Microcarcinoma of the uterine cervix: strong reactivity for p16INK4a in the nuclei of invading cells (×400). (c) Squamous carcinoma of uterine cervix, showing strong p16INK4a expression (×100). (d) Adenocarcinoma of uterine cervix. Strong p16INK4a expression; note the lack of stain in the normal endocervical epithelium (×400). (e) Argentophilic nucleolus organizer region (AgNOR) staining of a microinvasive carcinoma. Note the AgNOR dot areas from the neoplastic cells, bigger than the lymphocytes of the stroma (upper left) (×400).

malignant phenotype. Consequently, p16INK4a is overexpressed, so it can be used as a surrogate marker of progression of the lesion.[26]

The CIN1 are lesions of uncertain behavior, of which a percentage progresses to CIN2/CIN3, but no histological criteria allow differentiation between cases that progress or return in samples stained with HE.

The HPV type presents a very poor PPV. With the same consideration, its NPV is extremely high; a low-risk HPV type predicts a very low probability that a CIN1 evolves to cancer. There are chances, of course, for the patient to get a different type of virus throughout her life, and this is the basis of the annual visit to the gynecologist, to control and prevent uterine cervical cancer with a cytology study and colposcopy. Patients with an HSIL by cytology, confirmed by endocervical curettage, go to conization directly, treated surgically due to the high possibility of this lesion progressing to carcinoma. In patients with CIN1, the gynecologist's next procedure is not always clear, as the nature of the lesion, its persistence, the woman's age, previous results, and known risk factors (such as smoking, oral contraceptives, race, and others) need to be taken into account. In these cases, it is necessary to use a complementary assay to help predict the future of that lesion. In this work we used biopsy specimens with CIN1 diagnosis archived in the Pathology Laboratory of Clinical Hospital José de San Martín, Buenos Aires, Argentina, taken at two points, so we could compare the evolution of the lesions with the expression of two proliferation markers: p16INK4a and AgNOR.

Persistent infection is defined as the presence of HPV infection in two or more controls.[27] Some authors have questioned this concept, due to the different time periods in which the smears are obtained, and the different detection methods to establish the HPV infection.[28,29] To eliminate the probability of a new infection in our patients, the second biopsies were taken no longer than 8 months after the first one, and during the period between the two smears, cytology and colposcopy were carried out monthly. Twelve of the 20 CIN1 that regressed to metaplastic or non-dysplastic atypical epithelium showed a positive p16INK4a staining, but all of the second biopsies (those of benign epithelia) were positive for that marker. Thus, p16INKa is a

Table 1 Persistence, progression and regression of CIN1 and AgNOR areas/p16 scores

Group 1 AgNOR (μm^2)/ p16 score	Group 2 AgNOR (μm^2)/ p16 score *From CIN1 to CIN1*	Group 2 AgNOR (μm^2)/ p16 score *From LSIL to CIN2/CIN3*
2.45/0	3.08/2	5.04/2
2.01/0	3.60/2	4.06/2
2.95/2	3.34/1	3.47/2
1.73/1	5.30/2	4.13/2
2.79/2	3.86/2	4.00/2
2.19/2	3.02/2	**2.75**/2
2.65/2	5.52/2	4.58/2
2.60/1	3.21/1	4.30/2
2.92/2	4.43/2	5.22/2
2.92/2	4.51/2	3.96/2
1.71/0	3.07/1	4.06/2
1.80 /0	**2.15**/1	5.20/2
2.49/1	3.56/2	3.87/2
2.20/0	3.97/2	3.66/2
2.80/2	3.37/1	4.45/2
2.15/0	3.41/2	3.97/2
1.75/0	3.92/2	4.60/2
2.27/2	**2.87**/1	
1.83/0	4.56/2	
1.95/2	3.96/2	
	3.80/2	
	3.55/2	
	4.20/2	
	2.60/1	
	4.43/2	
	3.40/2	
	3.60/2	
	3.35/2	
	4.55/2	
	3.62/2	
	3.95/2	
	4.40/2	
	2.20/1	
	4.05/2	
	3.30/1	
	4.23/2	
	3.67/2	
	4.15/2	

Bold numerals indicate a low value in a lesion that progressed. AgNOR, argentophilic nucleolus organizer region; CIN1, Cervical intraepithelial neoplasia grade 1; LSIL, low squamous intraepithelial lesion.

highly sensitive marker for HPV infection but its specificity is low: 12 biopsies with high score for p16 cleared the lesion.

Wentzensen *et al*.[30] performed p16INK4a immunocytochemistry to locate cells that expressed p16INK4a in liquid-based cytology samples and used a nuclear scoring system based on several morphologic criteria

Table 2 Average and SD of AgNOR areas for Groups 1 and 2

Group	*n*	AgNOR average (μm^2)	SD	*P*-value
1	20	2.45	0.49	<0.0001
2	55	3.93	0.72	

AgNOR, argentophilic nucleolus organizer region; SD, standard deviation.

to interpret the degree of abnormality of these cells: score 1 (no abnormal nuclei), score 2 (slightly abnormal nuclei), score 3 (clearly abnormal nuclei) and score 4 (severely abnormal nuclei). Ten percent of the low-grade squamous intraepithelial lesion samples and 98% of the HSIL samples were scored as abnormal. This nuclear score system is a simple but reliable interpreter tool to identify patients with relevant lesions, thereby avoiding a large number of equivocal test results.

The implementation of p16INK4a immunohistochemistry is still limited by the lack of uniformity in the scoring systems, although their use would be of great benefit to women by decreasing the cytologic and histopathologic interobserver variability in the diagnosis of cervical preinvasive lesions.[29]

In CIN2/CIN3 and carcinomas, p16INK4a is expressed with high intensity, occupying the full epithelial thickness; but on reversing CIN1, it was detected with a focal and weak cytoplasmic staining (score 1) or occupying two-thirds or all of the epithelial thickness (score 2).

The PPV was 82%. It is not clear why the pattern of p16INK4a staining is cytoplasmic in the majority of the cells. This characteristic is observed also in cultures of cervical cells; in HT-3 cells, the specific staining appeared in both nuclei and cytoplasm, while in SiHa and C33a cells, it was exclusively cytoplasmic.[25]

AgNOR value as a marker of progression of CIN1 was reported in cervical cytology samples, so the variations in the areas of the NOR were observed in cells exfoliated from the surface of the epithelium.[31] Using the same regression (group 1) and persistent-progression (group 2) criteria, but considering the biopsies and not the exfoliated cells, we measured the NOR areas in parabasal layers, finding a greater difference between those two groups. The average of the AgNOR area in Group 1 was $2.44 \pm 0.49\ \mu m^2$, and in Group 2 it was $3.81 \pm 0.80\ \mu m^2$. The average AgNOR value of the normal epithelium was $1.81 \pm 1.10\ \mu m^2$ so, the cut-off we propose is $3.0\ \mu^2$. With this value, we can exclude all the CIN1 that reverted.

Samples with AgNOR areas less than 3.0 μ^2 (Group 1) returned in all cases, but Group 2, consisting of patients whose lesions persisted or progressed to CIN2/CIN3, showed AgNOR areas greater than 3.0 μ^2 in 50/55 cases (91%).

In the five patients in Group 2 with AgNOR areas of less than 3.0 μ^2 and persistent lesion, the second samples were taken in a very short time after the first one, therefore it is possible to consider that not enough time had passed for clarification of the lesion. Even including these five cases in Group 2, the PPV was 100%, and the NPV was 80%.

Conclusion

p16INK4a is a proliferation marker with low specificity as criterion of progression of CIN1, as it is expressed in a high percentage of returning lesions (PPV: 82%). Its lack of expression, in contrast, involves low proliferative potential (NPV 100%).

AgNOR might be a better marker of proliferation of CIN1, with PPV = 100%, which means that a value greater than 3.0 μ^2 indicates the persistence or progression of the lesion. Its NPV is 80%; therefore, a value of AgNOR area less than 3.0 μ^2 in CIN1 leaves a margin of doubt about the future behavior of the lesion.

The AgNOR could be used instead of p16INK4a, due to its high PPV and because it is a rapid, simple and inexpensive method. Its application as a complementary method of histology could predict the future of these lesions of uncertain behavior, even in old paraffin-embedded material, making it useful for historical samples. Due to these characteristics, this method is suitable for application in developing countries, where the molecular assays are currently still too expensive. The AgNOR fits the role of a screening method: high PPV, reproducibility, fast, and of low cost, and it could be included as a complementary assay with colposcopy and cytology. This could help the gynecologist to avoid further biopsies in the follow-up of patients with a diagnosis of CIN1, predicting the fate of this lesion.

Acknowledgment

Thanks to Dr Donald Richards for reviewing the language.

Disclosure

None declared.

References

1. Holowaty P, Miller AB, Rohan T *et al*. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999; **1**: 252–258.
2. Villa LL, Sichero L, Rahal P, To T. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. *J Gen Virol* 2000; **81**: 2959–2968.
3. Nasiell K, Roger V, Nasiell M. Behavior of mild cervical dysplasia during long term follow-up. *Obstet Gynecol* 1986; **67**: 665–669.
4. Walboomers JM, Jacobs MV, Manos MM. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999; **189**: 121–129.
5. zur Hausen H. Papillomaviruses and cancer: From basic studies to clinical application. *Nat Rev Cancer* 2002; **2**: 342–350.
6. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)* 2006; **110**: 525–541.
7. Duensing S, Münger K. Mini review: Mechanisms of genomic instability in human cancer: Insights from studies with human papillomavirus oncoproteins. *Int J Cancer* 2004; **109**: 157–162.
8. Sherman L, Jackman A, Itzhaki H, Stöppler MC, Koval D, Schlegel R. Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6 oncoprotein: Role of p53 inactivation. *Virology* 1997; **237**: 296–306.
9. Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev* 1997; **11**: 2090–2100.
10. Thomas M, Banks L. Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* 1998; **17**: 2943–2954.
11. Arbyn M, Paraskevaidis E, Martin-Hirsch P, Prendiville W, Dillner J. Clinical utility of HPV-DNA detection: Triage of minor cervical lesions, follow-up of women treated for high-grade CIN: An update of pooled evidence. *Gynecol Oncol* 2005; **99**: S7–S11.
12. D'Amico M, Wu K, Fu M. The inhibitor of cyclin-dependent kinase 4a/alternative reading frame (INK4a/ARF) locus encoded proteins p16INK4a and p19ARF repress cyclin D1 transcription through distinct cis elements. *Cancer Res* 2004; **64**: 4122–4130.
13. Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA. p16 (INK4a) expression correlates with degree of cervical neoplasia: A comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol* 2003; **16**: 665–673.
14. Wentzensen N, von Knebel Doeberitz M. Biomarkers in cervical cancer screening (Review). *Dis Markers* 2007; **23**: 315–330.
15. Derenzini M, Montanaro L, Treré D. What the nucleolus says to a tumour pathologist. *Histopathology* 2009; **54**: 753–762.
16. Gall JC. The genes of ribosomal RNA during oogenesis. *Genetics* 1969; **61** (Suppl): 121–132.
17. Goodpasture C, Bloom SE. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* 1975; **53**: 37–50.
18. Ploton D, Bendayan M, Adnet JJ. Ultrastructural localization of Ag-NOR proteins and nucleic acids in reticulated nucleoli. *Biol Cell* 1983; **49**: 29–34.

19. Singh M, Prasad S, Kalra N, Singh U, Shukla Y. Silver-stained nucleolar organizer regions in normal and dysplastic cervical lesions: Correlation with DNA ploidy and S-phase fraction by flow cytometry. *Oncology* 2007; **71**: 411–416.
20. Palaoro LA, Blanco AM, Gamboni M, Rocher AE, Rotenberg RG. Usefulness of ploidy, AgNOR and immunocytochemistry for differentiating benign and malignant cells in serous effusions. *Cytopathology* 2007; **18**: 33–39.
21. Stemberger-Papić S, Stanković T, Vrdoljak-Mozetic D *et al*. Morphometry and digital AgNOR analysis in cytological imprints of benign, borderline and malignant serous ovarian tumours. *Cytopathology* 2006; **17**: 382–389.
22. Rocher AE, Gonzalez A, Palaoro LA, Blanco AM. Usefulness of AgNOR technique and CEA expression in atypical metaplastic cells from cervical smears. *Anal Quant Cytol Histol* 2006; **28**: 130–136.
23. Massad LS, Eistein MH, Huh WK *et al*. ASCCP Consensus Guidelines Conference. 2012 updated consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors. *Obstet Gynecol* 2013; **121**: 829–846.
24. Kanai K, Nunoya T, Shibuya K, Nakamura T, Tajima M. Variations in effectiveness of antigen retrieval pretreatments for diagnostic immunohistochemistry. *Res Vet Sci* 1998; **64**: 57–61.
25. Volgareva G, Zavalishina L, Andreeva Y *et al*. Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. *BMC Cancer* 2004; **4**: 58–68.
26. Cuschieri K, Wentzensen N. Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 2536–2545.
27. Franco EL, Villa LL, Sobrinho JP, *et al*. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. *J Infect Dis* 1999; **180**: 1415–1423.
28. Koutsky LA, Holmes KK, Critchlow CW. A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. *N Engl J Med* 1992; **327**: 1272–1278.
29. van Bogaert L-J. P16INK4a immunocytochemistry/immunohistochemistry: Need for scoring uniformization to be clinically useful in gynecological pathology. *Ann Diagn Pathol* 2012; **16**: 422–426.
30. Wentzensen N, Bergeron CH, Cas F, Eschenbach D, Vinokurova S, von Knebel Doeberitz M. Evaluation of a nuclear score for p16INK4a-stained cervical squamous cells in liquid-based cytology samples. *Cancer* 2005; **105**: 461–467.
31. Misra JS, Chhavi N, Singh M, Srivastava AN, Das V. Assessment of potential of AgNOR counts as tumor marker in cervical carcinogenesis. *Diagn Cytopathol* 2008; **36**: 194–195.