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ORIGINAL ARTICLE

Comparative study of the expression of cellular cycle proteins in cervical intraepithelial lesions

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Abstract

Interaction of human papilloma virus oncoproteins E6 and E7 with cell cycle proteins leads to disturbances of the cell cycle mechanism and subsequent alteration in the expression of some proteins, such as $p16^{INK4a}$, cyclin D1, p53 and KI67. In this study, we compared alterations in the expression of these proteins during several stages of intraepitelial cervical carcinogenesis. Accordingly, an immunohistochemical study was performed on 50 cervical biopsies, including negative cases and intraepithelial neoplasias. The expression patterns of these markers were correlated with the histopathological diagnosis and infection with HPV. The $p16^{INK4a}$, followed by Ki67, showed better correlation with cancer progression than p53 and cyclin D1, which recommends their use in the evaluation of cervical carcinogenesis. These monoclonal antibodies can be applied to cervical biopsy specimens to identify lesions transformed by oncogenic HPV, separating CIN 1 ($p16^{INK4a}$ positive) and identifying high-grade lesions by an increase in the cellular proliferation index (Ki67). In this way, we propose immunomarkers that can be applied in clinical practice to separate patients who need a conservative therapeutic approach from those who require a more aggressive treatment.

Keywords: Cervical neoplasia; $P16^{INK4a}$; Cyclin D1; Ki67; p53

Introduction

Squamous cell carcinoma develops from well-defined, intraepithelial precancerous lesions, which have the potential to progress to invasive carcinoma if not detected and treated early. There is epidemiological evidence that persistent infection of the cervix with a high viral load of oncogenic types of human papilloma virus (HPV) plays a preponderate role in the development of uterine cervical carcinoma [4,13,27]. Moreover, the virus can be detected in almost all preneoplastic lesions and cervical carcinomas [33].

Persistent HPV infection may result in viral DNA integration into the host's genome [9]. During

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integration, viral DNA frequently breaks in region E1/E. This break results in loss of E2 function, increasing the main transforming genes, proteins E6 and E7 transcriptions [30], whose expression is an essential prerequisite to proliferation of cancerous cervical squamous cells [36]. The oncogenic potential of each HPV type seems to reflect the capacity of the viral oncogenes, E6 and E7, to interfere with the cellular cycle by degradation of the tumor suppressing gene p53's product and functional inactivation of the retinoblastoma gene pRb's product, respectively [1].

Biochemical evidence indicates that one of the first events in G1 is the synthesis of cyclins D, particularly D1 [29]. Cyclin D1 plays an important role in cell growth regulation, and its alterations may be involved in carcinogenesis. This cyclin interacts with CDK4 and CDK6, resulting in accumulation of these complexes. These kinases, which phosphorylate pRb, are necessary for the passage through the G1 phase of the cellular cycle [7,30], and their activities are regulated, in part, by CKIs, among which is $p16^{INK4a}$. Specific linking of $p16^{INK4a}$ protein with the CDKs, CDK4 and CDK6, inhibits the phosphorylation activity of the CDK-cyclin D1 complexes through the nuclear protein complex RB/EF [28]. The cyclin D/CDK4,6/$p16^{INK4a}$/Rb/E2F pathway plays a key role in control of cellular growth through integration with multiple mitogenic and non-mitogenic stimuli [29].

Even though the cellular cycle transitions depend on the CDKs' activity, other checkpoint controls exist. The gene p53, whose product is a 53 kD nuclear phosphoprotein, helps guarantee the stop in G1 so that the damaged DNA can be repaired before it replicates. Protein p53 works as a transcription factor, inducing the expression of proteins MDM and $p21^{CIP1}$. MDM2 catalyzes the destruction and inhibits the transcription of p53 [12]. The accumulation of $p21^{CIP1}$ inhibits the CDKs necessary for the start of DNA synthesis; therefore, the growth control pathways commanded by pRB (cyclin D/CDK4,6/INK4a/Rb/E2F) and p53 (p53/MDM2/$p21^{CIP1}$) are interconnected [12,29].

Antigen Ki67 is expressed during all phases of the cellular cycle, G1, S, G2, and M, of proliferating cells, but is absent in quiescent cells (G0). It is, therefore, a marker of cellular proliferation, which can be detected by mononuclear antibody [10,11].

Our objective was to evaluate comparatively the expression of cell cycle proteins $p16^{INK4a}$, cyclin D1, p53, and Ki67 in the epithelia of patients with normal cervices and those in various stages of cervical carcinogenesis and to detect a panel of markers that could be useful to identify patients at risk of developing invasive cancer.

Materials and methods

Informed consent for this study was obtained from 60 patients selected from women who presented an atypical transformation zone (ATZ) and an atypical cytology result or negative cytology with persistent ATZ ($\geqslant$6 months) at the State Center for Oncology–CICAN (State Health Authority) and at the Outpatient Clinic of Gynecology (Infectious Diseases/AIDS Unit) of the Professor Edgar Santos Hospital, Federal University of Bahia (UDAI-HUPES-UFBA) in the city of Salvador, state of Bahia in Brazil, between October 1999 and March 2001. The mean age of the patients was 37 years (ranging from 19 to 82 years).

Before colposcopy was performed, ectocervical and endocervical scrapes were made with a sterile brush for detection and typing of HPV by PCR (PCR-based line blot assay, first generation, Roche Molecular Systems). Biopsy was performed under colposcopic vision, and tissue samples were fixed in 4% buffered formalin, embedded in paraffin, and cut as 4 μm-thick sections for routinely stained hematoxylin–eosin slides and immunohistochemistry. Three pathologists examined all slides (CQ, ES and LF). The WHO classification was used for the histologic diagnosis.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections (4 μm-thick) were deparaffinized in xylene and rehydrated through a series of graded ethanols (100–70%). For antigen retrieval, slides were pretreated by boiling (90–95 °C) in citrate buffer (pH 6.0) for 20 min. The slides were then incubated in methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. After washing, sections were incubated overnight at 4 °C with primary antibody (Anti-human $p16^{INK4a}$/MTS1, MTM Laboratories, Clone E6H, 1:200; Anti-human cyclin D1, DAKO, Clone DCS-6, 1:100; Ki67, DAKO, Clone Ki-S5, 1:200; Anti-human p53 protein, DAKO, Clone DO-7, 1:50) in 53022DAKO diluent. After washing in PBS, the slides were incubated for 30 min with secondary antibody αIgG mouse coated in dextran polymer with several molecules of peroxidase (*DAKO Envision System*). After washing, color development was achieved with 3,3-diaminobenzine (DAB) as chromogen and hematoxylin counterstaining. Formalin-fixed, paraffin-embedded sections from invasive uterine cervix squamous cell carcinoma biopsies served as a positive control for $p16^{INK4a}$, lobular breast carcinoma for cyclin D1, colonic adenocarcinoma for p53, and breast carcinoma for Ki67. As a negative control, we used normal non-immune serum from the same source as the primary antibody. All slides (cases and controls) were submitted to the reaction in the same bath.

Positivity for the monoclonal antibodies

The reactions were evaluated in the area of the highest grade of CIN for all samples as previously described [23].

p16^{INK4a}: The reaction was considered positive when a chestnut-brown color was seen in the nuclei and cytoplasm. The intensity of the reaction was scored as negative (0), weak (1), moderate (2), and strong (3).

Cyclin D1: The nuclear reaction pattern for cyclin D1 was scored as negative (0), positivity in the basal layer as (1), and reaction of some cells above the basal layer as (2).

P53: Reaction was considered positive when the nucleus stained gold-brown. Nuclear positivity of up to 10% of the cells was scored as (0), nuclear positivity between 10% and 50% of the cells as (1), and nuclear positivity of over 50% of the cells as (2).

Ki67: A reaction was considered positive when a yellowish-brown color was observed in the upper two-thirds of the squamous epithelium. We considered positivity for Ki67 according to scores: positive cells only in the basal and parabasal layers (0), up to 20%, discrete proliferation (1); between 21 and 40%, moderate proliferation (2), and above 40%, intense proliferation (3).

Statistical tests: The tests used were Kruskal–Walls (KW), univariated and Spearman's correlations statistical techniques (Software R 2.0.1). For the comparative evaluation of the four markers used, the multiple correspondence analysis (MCA) statistical technique (SPAD3.5-Sistème Portable pour l'Analyse dês Données) was used to determine the associations between the several categories of markers (p16^{INK4a}, cyclin D1, p53, and Ki67) and their association with the histological grades.

Results

Among the 50 cases studied, there were 11 normal, 15 NIC1, 10 NIC2, and 14 NIC3 cases. Forty-one were positive, by PCR, for DNA-HPV, and 20 were HIV-positive.

Kruskal–Wallis' test showed a significant difference between the expression of cell cycle proteins p16^{INK4a}, cyclin D1, p53 and Ki67, and the various histological diagnoses ($p < 0.005$) as expressed in Table 1.

MCA is a descriptive, exploratory statistical technique that permits analysis of a database containing a large number of variables. It is an appropriate technique for studying the associations that occur among the various categories of a set of qualitative variables, since it reduces information to a bidimensional space in which a pair of dimensions (factorial axes) permits evaluation of the relationship between all these categories. The graphic illustration (factorial plan) provides a representation of multiple simultaneous associations among all the various strata of individual qualitative variables. These associations are identified by the proximity of these categories in the factorial plan.

Table 1. Positivity for markers p16^{INK4a}, cyclin D1, p53, and Ki67

	p16^{INK4a} (%)	Cyclin D1 (%)	p53 (%)	Ki67 (%)
Negative	9.1	100.0	0.0	9.1
CIN 1	66.7	80.0	26.7	40.0
CIN 2	90.0	10.0	0.0	100.0
CIN 3	100.0	0.0	42.9	100.0

Table 2. Self-values and inertia percentile of the factorial axes

Factor	Self-value	Inertia percentile (%)	Accumulated inertia percentile (%)
1	0.6067	26.96	26.96
2	0.3197	14.21	41.17
3	0.3045	13.53	54.70
4	0.2602	11.56	66.27
5	0.2390	10.62	76.89
6	0.2073	9.21	86.10
7	0.1643	7.30	93.40
8	0.1001	4.45	97.85
9	0.0483	2.15	100.00

According to Table 2, analysis of only the first and second factorial axes is suggested because, although the first four self-values surpass the mean eigenvalue, the inertia percentile decays in a moderate way, starting at the second factorial axis without showing great variation. The contribution percentile of these two first axes in the total variance of 41.17% permits us to affirm that the first two factorial axes comprising the principal factorial plan (Fig. 1) represent the best projection of the variables analyzed, providing unique, bidimensional visualization, i.e., a more reliable representation of the association between markers and histological degree, clearly showing what actually occurs with the data.

Analyzing the histological grade disposition (illustrative variable), we observed that the first factorial axis is more well-defined by this variable, which presents itself in the graph in an orderly fashion, from lower to higher severity, from left to right. Therefore, the first factorial axis characterizes the histological diagnosis. In this first factorial axis, we observed that the markers p16^{INK4a} and Ki67 distributed themselves in the same manner and also in the same manner as the histological grade. This is opposite to what occurs with cyclin D1. Variables whose category dispositions are found in the same directions

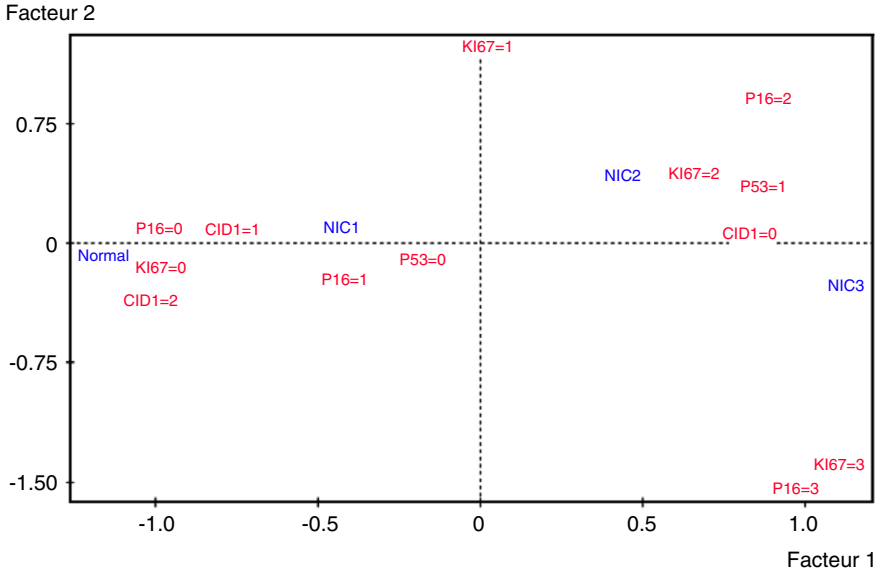

Fig. 1. Main plain-relation between histological grade and immunopositivity for $p16^{INK4a}$, cyclin D1, p53 and Ki67.

Table 3. Association (accumulated contribution) between markers and histological grade

Marker	Accumulated contribution (%)
$p16^{INK4a}$	84.7
Ki67	77.4
Cyclin D1	28.3
p53	9.6

Table 4. Spearman's correlations between markers $p16^{INK4a}$, cyclin D1, p53, and Ki67

	$p16^{INK4a}$	Cyclin D1	P53	Ki67
$p16^{INK4a}$	1.000	−0.518[a]	0.224	0.748[a]
Cyclin D1	−0.518[a]	1.000	−0.194	−0.653[a]
P53	0.224	−0.194	1.000	0.222
Ki67	0.748[a]	−0.653[a]	0.222	1.000

[a]Significant correlation: p-value (bilateral) <0.05.

Table 5. Predictive power of each of the markers according to histological grade (percentage of right results of immunomarkers $p16^{INK4a}$, cyclin D1, p53, and Ki67)

Histological grade	Global right results percentage (%)
$p16^{INK4a}$	58.0
Cyclin D1	52.0
p53	34.0
Ki67	54.0

are positively correlated, with a negative correlation occurring for the variables whose categories are situated in opposed directions [8]. The proximity of two markers in the same factorial axis also indicates that there exists a strong similarity and association between them. In this analysis, the accumulated contribution in the first two factorial axes can be used as a measure of association between positivity for these markers and severity of the histological diagnosis. Thus, Table 3 demonstrates that markers $p16^{INK4a}$ and Ki67 show a greater association with histological diagnoses.

Table 4 shows that the results of the MCAs were confirmed through Spearman's correlations.

Another statistical method used in this work was the univariated logistic regression technique, which aims to determine the predictive power of each of the four markers, having the histological grade as dependent variable. Through the univariated logistic regression models, adjusted for each of the four markers separately, we found with the global percentages of right results that the histological grades prognosticated by the model, using marker $p16^{INK4a}$, were those that were most equivalent to the real histological grades seen in this sample (Table 5). In this context, the second marker with best predictive power was Ki67, followed by cyclin D1. Marker p53 showed the least predictive result. These findings confirm what was observed in the MCA.

Discussion

In this study, we observed the association between the expression profiles of cellular cycle proteins $p16^{INK4a}$, cyclin D1, p53 and Ki67, and the progression from low-grade to high-grade CIN.

We analyzed the immunohistochemical positivity for $p16^{INK4a}$. Our results confirmed that pRb inactivation through p16/cdk-cyclin/Rb with increase in $p16^{INK4a}$ expression in HPV-transformed cells is an important mechanism for cervical carcinogenesis [5,16,25,26,31]. These studies verified the increased expression of $p16^{INK4a}$ in all cases of cervical dysplasia, while no expression was observed in normal or inflamed cervical mucosa [15,35].

We found an inverse expression of cyclin D1 in relation to the other studied cell cycle proteins, particularly $p16^{INK4a}$, which corroborates the inactivation mechanism of pRb by HPV E7 with increase of protein $p16^{INK4a}$ and reduction in expression of cyclin D1 protein [28]. These findings agree with the findings of Bae et al., who detected cyclin D1 in normal cervices and lack of expression in cervical lesions [3]. Skomedal et al. did not find expression of cyclin D1 in most (87%) of the low-grade lesions infected with oncogenic HPV, while observing nuclear expression in the basal, parabasal, and, occasionally, in the intermediary layers in 92% of the low-grade lesions infected with low-risk HPV [30].

In the uterine cervix, the immunohistochemical results for detection of p53 protein are contradictory [6,19,32]. Increased expression of p53 is detected in invasive and *in situ* carcinoma [6,22] while dysplastic and normal tissues are negative [7]. We noted greater expression of p53 in CIN 3 cases, which agrees with the findings of Cooper et al. [5], who observed nuclear positivity in 86% of the carcinomas with the same antibody used in this study. Other studies do not show any difference in expression with CIN progression [18,32].

Several studies have indicated that Ki67 is a relevant prognostic marker in the study of human tumors. Immunopositivity for Ki67 reflects the relation among proliferative action, CIN grade, and HPV expression [17,18,24]. We observed varied expressions in negative cases, which agrees with other reports [2,17,24]. This can be explained by hormonal influence, interfering with the number of proliferating cells [17]. Resnick et al. [24] found that the proportion of parabasal cell varied between 10% and 50%, and they did not find a correlation between frequency of stained parabasal cells in the normal epithelium and CIN. In other words, a change occurs in the expression pattern of this antigen in the basal and parabasal cells (normal epithelium, without CIN) as compared with intermediate layer (CIN1) and superficial layers (CIN2 and 3) [2,18].

Some authors have simultaneously studied the expression of cell cycle proteins by immunohistochemistry in cervical carcinogenesis. Skomedal et al. [30] studied several cell cycle proteins, including p53 and cyclin D1, and did not find any difference in aberrant expression or co-expression of any of these proteins according to histological type, degree of differentiation of the neoplasms, and patient survival. However, in relation to CINs, Keating et al. [14] found that the result of immunohistochemistry with Ki67, cyclin E, and p16 correlated with histological diagnosis, with positive scores of 68.4%, 96.7%, and 100% in low-grade lesions, and 94.75%, 91.65%, and 100% in high-grade lesions, respectively. Kruse et al. showed that Ki67-Si90 > 0.57 immunoquantification is an unfavorable prognostic sign, but only if Rb in the deep epithelium is low [18]. Wang et al. showed that increased $p16^{INK4a}$, p14ARF, and decreased or stable p53 expression are associated with progression of cervical disease [34]. We found equivalent results in both correspondence analysis and logistic regression models, and that monoclonal antibody $p16^{INK4a}$, followed by Ki67, showed better results than p53 and cyclin D1 in relation to the histological diagnoses, thus being considered an important component in a panel of immunomarkers for cervical lesions.

We noted a relation between $p16^{INK4a}$ and Ki67 expression and the severity of histological grade, and an inverse relation between cyclin D1 and progression of cervical carcinogenesis. As for p53, we did not observe a clear association between its expression and progression of carcinoma.

As for the presence or absence of HPV, we verified that there was a difference between HPV-negative patients, who showed low positivity for $p16^{INK4a}$, p53 and Ki67, and HPV-positive patients who showed a higher positivity of these markers. Cyclin D1 behaved in an inverse way.

For the diagnosis of most preneoplastic and neoplastic lesions of the uterine cervix, the conventional morphological exams are sufficient. Under ideal circumstances, the CIN grade assessed by the three methods (colposcopy, cytology, and histopathology) should be the same. However, discordance of more than one grade between cytology and biopsy reflects accentuated discrepancy, which will frequently have its repercussion in the treatment and follow-up of the patient [20].

To overcome these limitations, it becomes necessary to identify biomarkers with a better predictive value for the presence of CIN because recognition of the molecular pathology of the disease can help in prognosis and treatment. In addition, histological examination does not permit identification of viral type and oncogenic potential, and the cellular changes do not reflect viral integration.

We believe that the results from this study recommend the use of monoclonal antibodies $p16^{INK4a}$ and Ki67 for the evaluation of some cervical biopsy cases: $p16^{INK4a}$ separates the HPV-negative cases or those infected with low-risk HPV from those infected with high oncogenic risk HPV in which transformation has already occurred. Consequently, this marker can potentially differentiate those lesions with an increased risk of progression to high-grade lesions, which is in agreement with Negri

et al. [21]. A positive reaction for Ki67 is an important indicator of a high-grade lesion, but is less precise with low-grade lesions, reflecting better the proliferative activity in neoplasias.

It seems reasonable to apply these monoclonal antibodies to some cervical biopsies cases, aiming to identify lesions transformed by oncogenic HPV, separating the CIN1 with progression potential ($p16^{INK4a}$), and identifying high-grade lesions by progressive increase of the cellular progression index (Ki67). Also, this application may help reduce subjectivity in the interpretation by pathologists, particularly in initial or doubtful lesions.

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