

The Use of Molecular Markers (p16, Ki-67 and E-Cadherin) in Uterine Cervical Biopsies

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Abstract: *Introduction:* Cervical cancer is related to the Human Papillomavirus (HPV). The E7 viral DNA sequence induces the start of DNA synthesis of infected cell, releasing protein p16. The sequence E6 inhibits apoptosis, with prolonged survival of cells heavily damaged and changed, with inhibition of p53 protein and increasing of protein Ki-67. In those injured cells, the molecules are reduced to join the cell membrane, the type E-cadherin.

Aim: To study the expression of p16 protein in: normal epithelium cervical, cervical lesions, pre-invasive (CIN) persistent and no persistent lesions and invasive carcinoma of the cervix and to correlate with the expression of Ki-67 and E-cadherin.

Patients and Methods: 54 uterine cervix biopsies were selected and submitted to immunohistochemical study, with biomarkers p16, Ki-67 and E-cadherin.

Results: 1 CIN I (27.9%) and CIN II (47.9%) had lower expression of p16 than in CIN III (73.5%) and invasive carcinoma (72.7%) ($p < 0.0005$). For Ki-67, invasive carcinoma (57.8%), had a higher expression when compared to CIN I (35.6%), CIN II (51.9%) and CIN III (40.9%) ($p = 0.005$). E-cadherin expression in invasive carcinoma (46.2%) was lower than in CIN III (56.0%), CIN II (77.4%) and CIN I (82.2%) ($p < 0.0005$) and, normal epithelium had the greatest E-cadherin expression (89.1%). In persistent and no persistent CIN there was no difference in the expression of the biomarkers, with p16 presenting $p = 0.50$, Ki-67, $p = 0.91$ and the E-cadherin a $p = 0.43$ value.

Conclusions: The use of p16, Ki-67 and E-cadherin biomarkers in cervical biopsies with difficult diagnosis could help in the early diagnosis of malignant lesions and support adequate treatment, 2. There is no association between the diagnosis of the biopsy and the persistence of the cervical lesion and, 3. The used biomarkers don't differentiate between persistent CIN and no persistent lesions.

Keywords: p16, ki-67, E-cadherin, uterine cervical cancer, persistent cervical intraepithelial neoplasia.

INTRODUCTION

Epidemiological evidence shows that breast and genital cancers are the most frequent cancers among women worldwide [1]. It is estimated that around 500,000 women per year develop cancer of the uterine cervix worldwide. In Brazil, the National Cancer Institute (INCA), estimates, for 2008 and 2009, approximately 466,730 new cases per 100,000 inhabitants. In our country, cancer of the cervix is the second most common malignancy among women with an estimate of 19,000 new cases following only to breast cancer, and is the fourth cause of death by cancer in women [2].

The epidemiological profile of the disease shows that it is related to sexual activity, and associated to HPV infection [3]. The high risk HPV types 16 and 18 are the most prevalent, representing 59.8% and 15%, respectively, in cases of

invasive cancer [4, 5]. A persistent high-risk HPV infection is considered not only a risk factor, but also as a prerequisite for the development of cervical cancer [6].

Recent studies show that the presence of HPV DNA in more than 99.7% of cases of cervical intraepithelial neoplasia (CIN) used the technique of polymerase chain reaction (PCR). It is well established that HPV infection is the central and causal factor of cervical cancer [4]. Risk factors such as age of initiation of sexual activity, number of sexual partners, number of children, smoking, low socio-economic-cultural status and dietary deficiency of some elements are part of the natural history of this disease [1].

Therefore, cervical cancer represents a real public health problem, and is directly linked to the degree of underdevelopment of countries. It is one of the best examples of a cancer that can be prevented. Knowledge of the natural history of cervical cancer, which usually presents with a relatively slow progression, and the widespread use of screening methods for the detection of precursor and early stage lesions have permitted efficient secondary prevention in recent decades. Currently, primary prevention, through the use of vac-

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cines against human Papillomavirus (HPV) has been demonstrated [4, 7-9].

According to World Health Organization (WHO, 2008) [10] classification of the cervical uterine tumor, the precursor lesions of cervical cancer are classified as CIN I, CIN II and CIN III. Because there is a difficulty in diagnosis of such precursors, they can also be referred to as high and low grade lesions. However, sometimes in small biopsy it is quite difficult to differentiate lesions of low and high grade, and this diagnosis is very important to patient's treatment.

Protein P16

Several studies have highlighted the role of p16 as a marker of cervical carcinoma P16 expression is associated with the progression of disease and is directly related to the presence of HPV [4,7,11,12].

P16 belongs to the group of cyclin-dependent kinase Cdk4/6 inhibitors and is encoded by tumor suppressor gene *INK4a*. Gene *INK4a* plays an important role in the regulatory pathway Cdk-Rb-E2F. The product of this gene, p16INK4a, prevents pRb phosphorylation by inactivating Cdk4/6; pRb keeps on binding E2F transcription factors and as a result cells stay in G1 phase and do not pass to DNA replication. In cervical lesions induced by HPV, viral oncoprotein E7 interacts with pRb and inactivates it. As a result, the regulatory pathway Cdk-Rb-E2F is disrupted and inactivated pRb passes the cell cycle checkpoint G1/S without any obstacle. As a response, an overexpression of p16 occurs. In turn, p16INK4a protein can be a marker of premalignant and malignant cervical epithelium cells. Functionally active gene *RB* is shown to be able to negatively regulate the expression of *INK4a* on a transcriptional level, but the details of this negative feedback loop are still obscured [13-17].

In short, p16 expression, which can be detected immunohistochemically, is directly related to the presence of HPV [8]. Thus, this protein can be used as a biomarker that can add significant diagnostic precision in the assessment of CIN lesions [7, 12].

Ki-67

The Ki-67 is a marker of protein, non-histonic of cell proliferation, and is expressed in all phases of the cell cycle, except in G0 [18, 19]. This protein has a function of growth in human tumor, and expression of its marker could suggest the degree of malignancy [20-22].

The interaction of E6 and E7 HPV DNA in the host cell disturbs the cell cycle, expressing themselves by the abnormal expression of proteins, including the Ki-67 [13].

Some studies have shown that the Ki-67 immunohistochemistry positivity demonstrates the increasing proliferation in low and high grades of intraepithelial lesions [23]. In others, the results of analysis are consistent with a strong relationship between Ki-67 and p16, in the recognition of HPV-associated pre-invasive cervical lesions [13].

E-Cadherin

Cadherins are glycoproteins of 120 to 130 kDa that are involved in the cell adhesion and received this name due to the need of calcium (Ca) in order to link to them. The firm

intercellular adhesion attributed to the function of adhesive interactions plays a crucial role in tissue formation, since its involvement consists of an important biomarker for tumor development [24-28].

The squamous cells of cervix epithelium are strongly attached to each other and to the basement membrane through a large number of molecules of adhesion. Thus, E-Cadherin is one of the key molecules of adhesion that define the architecture and differentiation of keratinocytes in that epithelium. It is known that in intra-epithelial cervical cancer, there is a change in the expression of these molecules [25].

This suggests that the decrease or loss of expression of E-cadherin can be correlated with aggressive behavior and progression of cancer [24]. Roa *et al.* [29] consider Cadherins as the most important mediators of cell adhesion molecules and showed that the loss of this molecule in tumor tissues lines determines the ability to invade the collagen of tissues.

It is presumed that this down-regulation reduces the capacity of cells to adhere each other and facilitates their shut-down of primary tumor and metastasis. Therefore, the decrease in the expression of E-cadherin seems to be a useful parameter in evaluating the potential for malignancy of cervical cancer [24].

Dursun *et al.* [30] concluded that reduced expression of E-cadherin is significantly associated with overall survival and disease-free survival in the patients with cervical carcinoma, serving as an indicator of aggressive clinical behavior and could suggest the use of adjuvant therapy in early stages of the disease.

Histological Diagnosis

Although there are histological criteria for the diagnosis of cervical lesions [31], it is often vulnerable due to the size of the sample, and thus undermining the subsequent clinical conduct. As the cost of immunohistochemical study is cheaper than PCR for HPV, it would be interesting to find immunomarkers for different degrees of CIN. Thus, the aim of our work is to study the immunohistochemical expression of p16, Ki-67 and E-cadherin proteins in benign lesions, pre-invasive and invasive carcinoma of uterine cervix, and to correlate the expression of these markers together in the cases with difficult interpretation, to help in diagnosis and prognosis of cervical lesions.

MATERIAL AND METHODS

Samples

Prior to the beginning of this work, this study was approved by the Research Ethics Committee of the FAMERP, file number 001-000494/2007, following the legal procedures.

Women submitted to cervical biopsies between 2004 and 2007 were selected. We evaluated the morphological changes in histological sections stained by hematoxylin and eosin (HE), according to the severity of cervical lesion (normal cervix, with CIN I, II or III and with invasive carcinoma of the cervix). Immunohistochemical study was performed for p16, Ki67, and E-cadherin.

The immunohistochemistry technique for E-cadherin (NCL-E, Novocastra, clone 36B5), Ki-67 nuclear antigen (NCL-Hi-67, MM1, Novocastra) and p16 (NCL-p16 - 432, Novocastra) was then used and has been summarized as it follows: the 4 μ m cuts were dew axed, underwent antigenic recuperation, suffered peroxidase block and then were incubated into primary antibodies: ki-67 antigen mouse monoclonal antibody, dissolved in 1 : 600, the cadherin-E mouse monoclonal antibody dissolved in 1 : 200 and the p16 mouse monoclonal antibody dissolved in 1 : 50. The incubation with the biotinylated anti-Ig antibody or secondary antibody was used, which is specific for animal species, whose primary antibody was made (kit DAKO LSAB-labeled streptavidin biotin) for Ki-67 and cadherin-E, and for p16, secondary antibody was used (kit NOVOLINK-Polymer Detection Systems), dissolved in PBS for 30 minutes at 37°C in a humid room. Next, streptavidin biotin peroxidase complex incubation was made (Kit, peroxidase-DakoCytomation, Carpinteria, CA, USA). For the revelation, the cromogenic diaminobenzine subtract was used and was stained with hematoxylin of Harris.

Quantification of Immunohistochemical Results and Statistical Analysis

To evaluate the marker positivity, we counted at least 500 cells per case, in a blind manner. Positivity was nuclear

for Ki-67, nuclear and cytoplasmic for p16, and cytoplasmic membrane for E-cadherin in light microscopy, with a magnification of 400 \times . We made a quantification of the results by determining the index of positivity (number of cells marked by the antibody (p16, Ki-67 or E-cadherin) divided by the number of cells counted per sample).

Through the patients' electronic handbook, we selected 12 cases that presented persistence of the lesion in the last four years (April of 2003 to March of 2007), followed by periodic biopsy and/or cervical Pap smear. We compared these patients' initial diagnoses with the group of eight patients that didn't present recurrence of the disease in the same period of time, followed by cytological examination.

Statistical analysis was made with the use of non-parametric tests (Median, ANOVA and tabled statistics).

RESULTS

The 54 selected biopsies were classified as normal epithelium (5 cases), 12 cases of CIN I, 12 of CIN II, 13 of CIN III or squamous cell carcinoma in situ, and 12 cases of invasive squamous cell carcinoma of the uterine cervix (SCC).

The women's age ranged from 22 to 90 years, with an average of 45.74 years (median = 45). Figs. (1-5) show examples of hematoxylin and eosin stain, and p16, Ki-67 and E-cadherin positivity in different diagnosis.

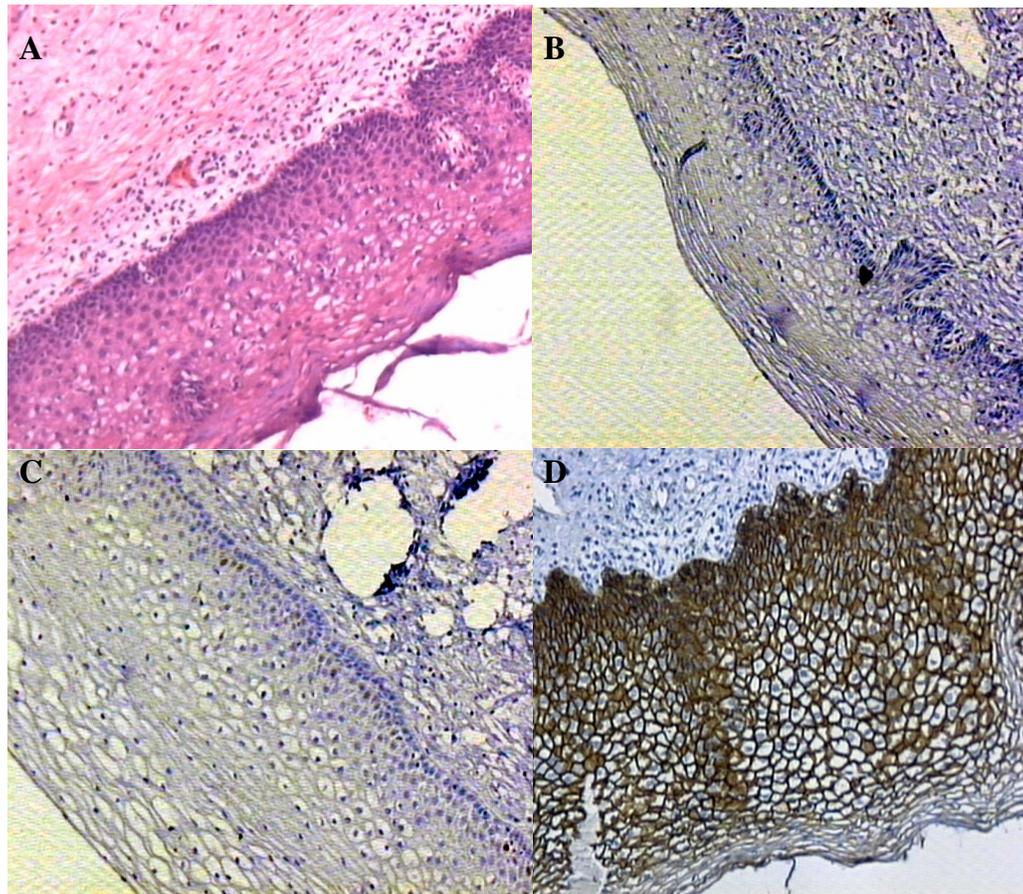


Fig. (1). Photomicrography of normal uterine cervix: (A) HE stain (100X); (B) p16 antibody (100X); (C) Ki-67 antibody (100X) and (D) E-cadherin antibody (100X). The number of positive cells for E-cadherin had the greatest expression (89.05%) when compared with the cervical lesions. There was no p16 expression and a low positivity for Ki-67 (6.6%).

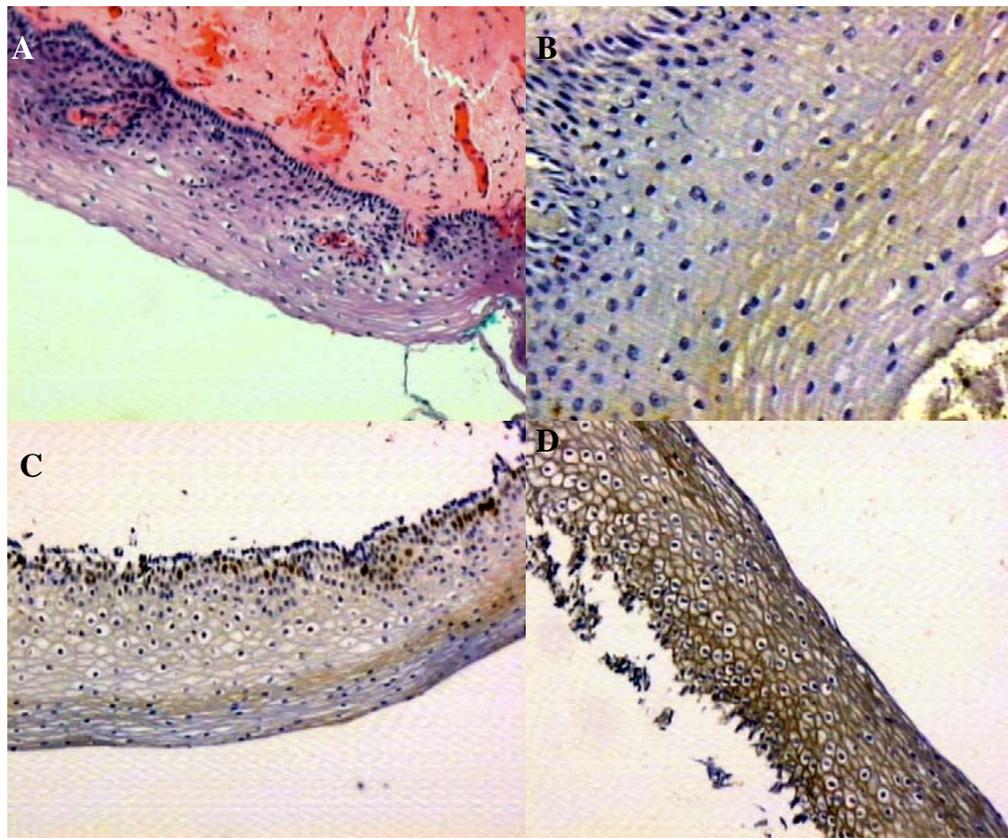


Fig. (2). Photomicrography of CIN I (Cervical intraepithelial neoplasia grade I): (A) HE stain (100X); (B) p16 antibody (200X); (C) Ki-67 antibody (100X) and (D) E-cadherin antibody (100X). The number of positive cells for p16 (27.94%) and Ki-67 (35.6%) was lower than for E-cadherin (82.18%).

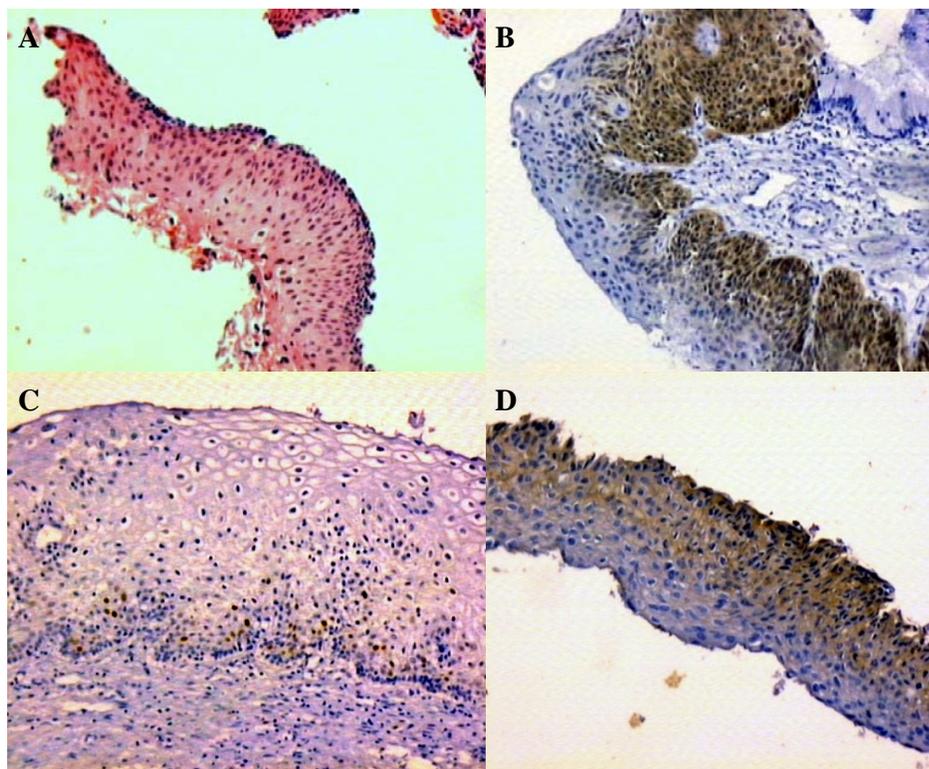


Fig. (3). Photomicrography of CIN II (Cervical intraepithelial neoplasia grade II): (A) HE stain (100X); (B) p16 antibody (100X); (C) Ki-67 antibody (100X) and (D) E-cadherin antibody (100X). The antibody E-cadherin had a higher expression (77.39%) when compared with p16 (47.93%) and Ki-67 (51.9%).

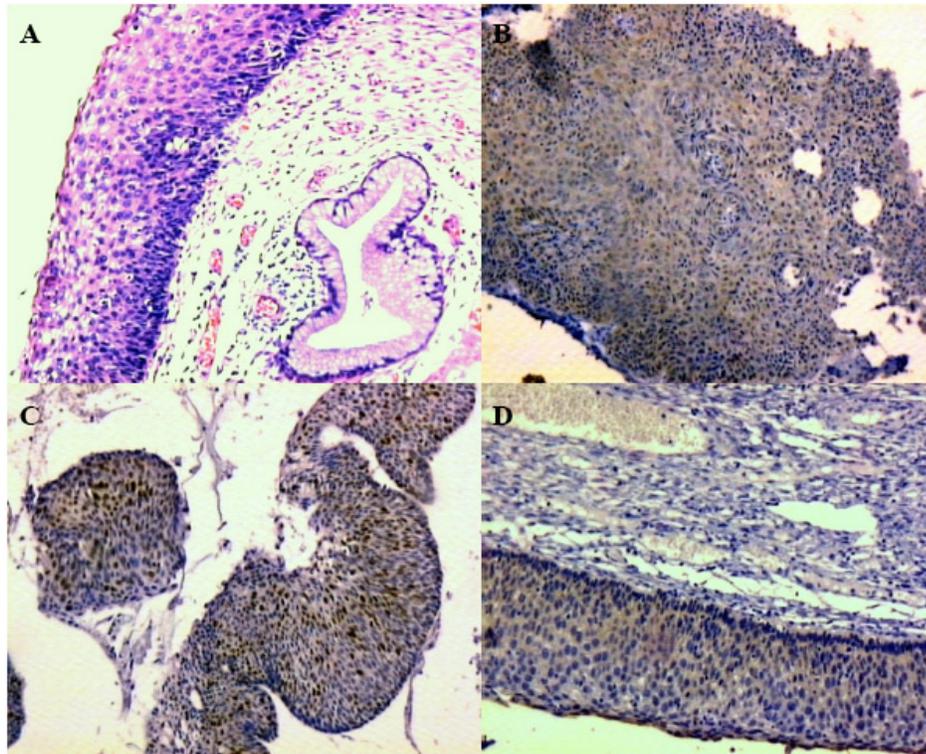


Fig. (4). Photomicrography of CIN III (Cervical intraepithelial neoplasia grade III): (A) HE stain (100X); (B) p16 antibody (100X); (C) Ki-67 antibody (100X) and (D) E-cadherin antibody (100X). The antibody p16 had a higher expression (73.47%) when compared with Ki-67 (40.9%) and the E-cadherin (55.96%).

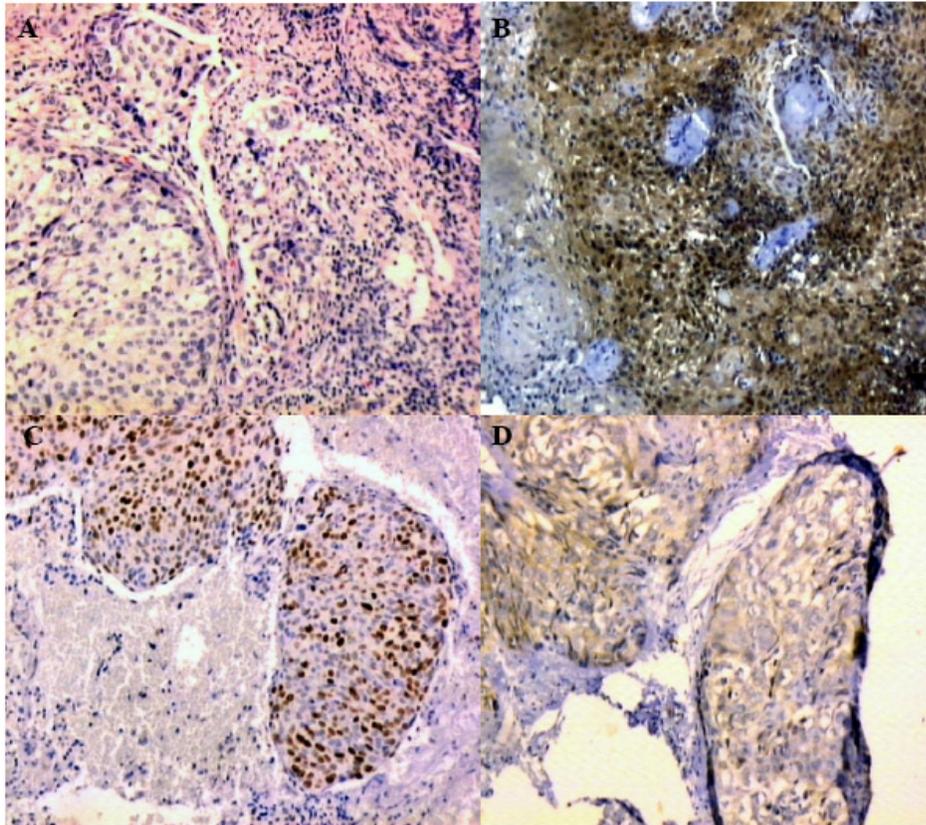


Fig. (5). Photomicrography of Invasive Carcinoma: (A) HE stain (200X); (B) p16 antibody (100X); (C) Ki-67 antibody (100X) and (D) E-cadherin antibody (100X). The number of positive cells for E-cadherin (46.15%) and Ki-67 (57.8%) was lower than for p16 antibody (72.70%).

P16 versus Diagnosis

Fig. (6) shows the distribution of positivity for p16 in the different groups in order to compare with the diagnosis. The histological sections of normal uterine cervix showed no expression of p16.

Through ANOVA test, we observed a statistically significant difference between the average percentages in the groups. CIN I (27.94%) and CIN II (47.93%) presented expression of p16 lower than CIN III (73.47%) and invasive carcinoma (72.70%) ($p < 0.0005$).

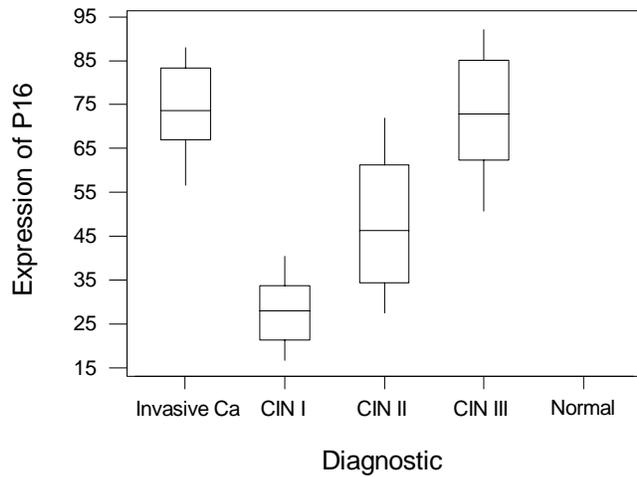


Fig. (6). Expression of p16 in different types of cervical lesion. There is an increase of p16 according to the degree of malignancy of injuries ($p < 0.0005$). Normal tissue did not present p16 expression. Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial neoplasia grade II, CIN III - Cervical intraepithelial neoplasia grade III.

Ki-67 versus Diagnosis

Fig. (7) shows the different positivities to the Ki-67 evaluated in the groups. There was statistically significant difference only between the median for the normal group (6.6%) with the neoplastic lesions. Invasive carcinoma (57.8%), was highly positive for Ki-67 when compared to CIN I (35.6%), CIN II (51.9%) and CIN III (40.9%), but there was no statistically significant difference eas found between them ($p = 0.005$).

E-Cadherin versus Diagnosis

Fig. (8) shows the distribution of expression of E-cadherin in groups (control and study), compared to the diagnosis.

Positivity for E-cadherin in the cases diagnosed with CIN III was the lowest observed among the pre-neoplastic lesions (55.96%), whereas in cases of CIN I was 82.18%, for CIN II, 77.39% and in the control group, 89.05%. There was statistically significant difference when comparing the positive cases of invasive carcinoma (46.15%) with CIN I and CIN II ($p < 0.0005$).

The Persistence of the Cervical Intraepithelial Neoplasia

As for the persistence of the cervical intraepithelial neoplasia, we found in the 20 selected patients, 40.0% with diagnosis of CIN I, 30.0% of CIN II and 30.0% of CIN III.

We observed that there was no statistical correlation between the degree of CIN and the persistence of the lesion ($p = 0.27$) (Table 1).

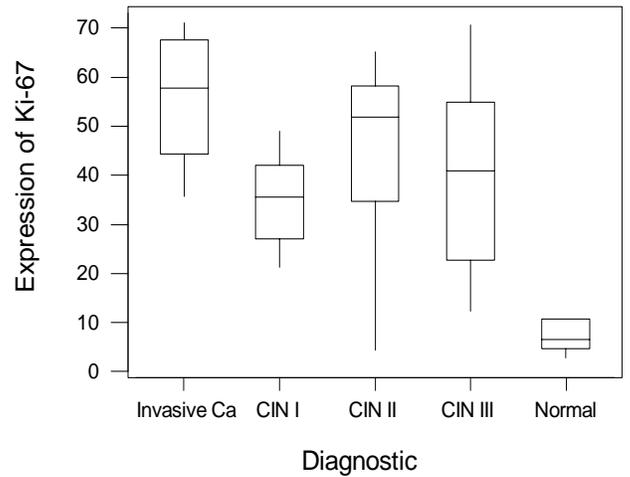


Fig. (7). Positive for Ki-67 in different types of injury of the uterine cervix. Ki-67 is higher expressed in neoplastic groups than in normal cervical biopsies ($p = 0.005$). Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial neoplasia grade II, CIN III - Cervical intraepithelial neoplasia grade III.

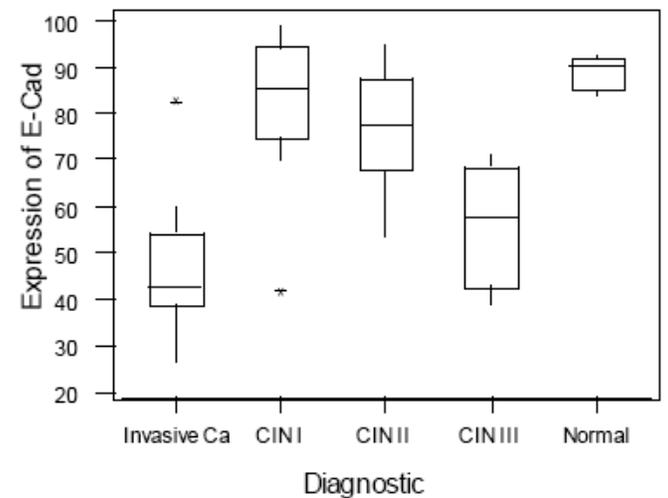


Fig. (8). Expression of E-cadherin in different types of injury of the uterine cervix. There was statistically significant difference when comparing the positive cases of invasive carcinoma (46.15%) with CIN I and CIN II ($p < 0.0005$). Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial neoplasia grade II, CIN III - Cervical intraepithelial neoplasia grade III.

In persistent and no persistent CIN there was no difference in the expression of the biomarkers, with p16 presenting $p = 0.50$, Ki-67, $p = 0.91$ and the E-cadherin a $p = 0.43$ value (data not shown).

DISCUSSION

In our study, we demonstrated an increased expression of the protein p16 from CIN I to invasive squamous cell carcinoma (SCC). For Ki-67 and E-cadherin, expression was direct and inversely related, respectively, with p16. This fact could help in the differential diagnosis between the lesions

Table 1. Analysis of Biopsy for No Persistent and Persistent HPV in Different Types of Injury of the Uterine Cervix. There was No Statistical Correlation Between the Degree of CIN and the Persistence of the Lesion ($p = 0.27$)

| HPV | BIOPSY | | | |
|--------------------------|--------|--------|---------|-------|
| | CIN I | CIN II | CIN III | Total |
| No Persistent HPV | 2 | 2 | 4 | 8 |
| Row % | 25,0 | 25,0 | 50,0 | 100,0 |
| Col % | 25,0 | 33,3 | 66,7 | 40,0 |
| Persistent HPV | 6 | 4 | 2 | 12 |
| Row % | 50,0 | 33,3 | 16,7 | 100,0 |
| Col % | 75,0 | 66,7 | 33,3 | 60,0 |
| Total | 8 | 6 | 6 | 20 |
| Row % | 40,0 | 30,0 | 30,0 | 100,0 |
| Col % | 100,0 | 100,0 | 100,0 | 100,0 |

Single Table Analysis

| | | |
|-------------------|-----------|----------|
| Chi-Square | df | p |
| 2,6389 | 2 | 0,2673 |

Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial neoplasia grade II, CIN III - Cervical intraepithelial neoplasia grade III.

and may be a good marker to detect risk of developing cervical cancer in women infected by HPV.

All cases of SCC and CIN III in our study had an overexpression of p16. Similar evidence was obtained by Benevolo [11], Volgareva [31] and Tringler [32], which reported a greater expression of p16 in a large percentage of pre-malignant lesions and invasive SCC. Several studies have shown an increase in the expression of p16 protein in accordance to the degree of malignancy of lesions, showing to be a great marker specific for pre-malignant and malignant lesions [4, 32].

Moreover, our cases of normal cervix showed no expression of p16. Maehama and colleagues [33] reported that they found 10.6% of positive for HPV in women with normal cytology smear, using PCR technique. Based on that report, we expected to find some expression of p16 protein in this group of patients, which may not have happened because of the small number of cases studied, or non-viral integration in the genome of the host [16, 18].

In our study we observed that the associated use of biomarkers p16 and E-cadherin together is a good combination for diagnosis of cervical lesions. In respect to the use of Ki-67, there was no significant difference in invasive and pre-invasive lesions (CIN I, II and III). However, According to Kruse *et al.* [20], Ki-67 is a good diagnostic marker for CIN III, however, the reproducibility for CIN I and CIN II was not satisfactory. Our negative results could be due the small number of samples used in our study.

Several authors, such as Benevolo [12], Keating [13], Abeer [16], Volgareva [31], Tringler [32], Longato Filho [34] and Walts [35], showed similar results using these markers and concluding that p16 is the defining role in early detection of cervical cancer and Ki-67 can be used as a factor of prognosis. For Keating (2001) [13], as far as Ki-67 is a good combination with p16 for diagnosis, E-cadherin expression also proved to be a substitute for Ki-67, supplementing the p16 marker for HPV in pre-neoplastic lesions and invasive cervical squamous carcinomas.

The median age of women in our study was 45.74 years, ranging from 22 to 90 years. Of these, 37 (68.52%) had a diagnosis of pre-invasive lesions. It is known that the incidence of cancer of the cervix is usually at its peak age group increased slightly [2], which is consistent with data from other studies [36-39].

The infection for HPV frequently happens and it ends quickly in most of the young women that begin sexual relationships, although HPV 16 persistent can progress to CIN III earlier than the non-persistent lesions [40]. Women that live below the poverty line have larger probability of being positive for HPV of high risk [41]. Many authors [42, 43] tell that in the persistent cases there is an association with HPV of high risk, what was not possible to evaluate in our work.

We did not perform the PCR test to study the HPV status in cervical lesions due to the high cost of this exam. However, p16 protein expression is considered a specific marker for this virus [4, 7] and can be used to differentiate patients with low grade lesions from others with high grade, that require a more aggressive treatment.

Although our work has shown similar results to those of international literature, there is no similar data with the Brazilian population using the three markers concurrently. Due to low cost, imunohistochemistry instead of PCR, is an economically feasible method, and thus can be easily performed in laboratory practice.

Thus, we suggest the use of biomarkers p16, Ki-67 and E-cadherin together to the diagnosis and prognosis of cervical lesions in order to help to differentiate lesions of low and high grade in difficult biopsies, but they are not helpful to differentiate between persistent and no persistent CIN.

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