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Dissertação apresentada à Faculdade de Medicina de São José do Rio preto para obtenção do Título de Mestre no Curso de Pós-Graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientadora: Prof. Dra. Patrícia Maluf Cury
Co-orientadora: Prof. Dra.Jane Lopes Bonilha

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Munhoz, Natália Gaspar

O uso dos marcadores moleculares (p16, Ki-67 e E-caderina) em biópsias uterinas cervicais / Natália Gaspar Munhoz.
São José do Rio Preto, 2009
30 p.; 29,5 cm

Dissertação (Mestrado) – Faculdade de Medicina de São José do Rio Preto
Eixo Temático: Medicina e Ciências Correlatas

Orientadora: Profª Drª Patrícia Maluf Cury
Co-orientadora: Profª Drª Jane Lopes Bonilha

1. Marcadores Biológicos de Tumor; 2. Biópsia; 3. Genes p16; 4. Antígeno Ki-67; 5. Caderinas; 6. Displasia do Colo do Útero; 7. Neoplasia Intra-Epitelial Cervical.

SUMÁRIO

1 INTRODUÇÃO

1.1 Epidemiologia

Com aproximadamente 500 mil casos novos por ano no mundo, o câncer do colo do útero é o segundo tipo de câncer mais comum entre as mulheres, sendo responsável pelo óbito de, aproximadamente, 230 mil mulheres por ano. Sua incidência é cerca de duas vezes maior em países menos desenvolvidos, se comparada à dos mais desenvolvidos. A incidência por câncer do colo do útero torna-se evidente na faixa etária de 20 a 29 anos, e o risco aumenta, rapidamente, até atingir seu pico geralmente na faixa etária de 45 a 49 anos. Em países desenvolvidos, a sobrevida média estimada em cinco anos varia de 59% a 69%. Nos países em desenvolvimento, os casos são encontrados em estágios relativamente avançados, e, conseqüentemente, a sobrevida média é de cerca de 49% após cinco anos. No Brasil, a estimativa para 2008, o Instituto Nacional do Câncer (INCA), é de cerca de 466.730 novos casos de câncer por 100.000 habitantes sendo que 19.000 são de colo uterino.[1]

Existem alguns fatores de risco para desenvolver o câncer cervical e ter o contágio pelo HPV (Papilomavírus Humano), como por exemplo; início precoce da vida sexual, particularmente antes dos dezesseis anos de idade; parceiros sexuais múltiplos; existência de doença sexualmente transmissível e baixo nível sócio-econômico. É necessária a existência do HPV, mas não é suficiente, é preciso que se tenha a intervenção de alguns co-fatores para se desencadear o processo neoplásico, como por exemplo; raio x; cigarro; uso de hormônios esteróides; vírus Herpético; vírus HIV; alta paridade; supressão do sistema imune; algumas bactérias como as clamídias e alguns fatores dietéticos e nutricionais como a deficiência de vitaminas A e D.[2,3]

Os HPVs de alto risco dos tipos 16 e 18 são mais prevalentes e representam 59,8% e 15% respectivamente, nos casos de câncer invasivo.[4,5] A persistência da infecção por HPV de alto potencial oncogênico é considerada não apenas um fator de risco, mas também um pré-requisito para o desenvolvimento de câncer cervical.[6]

Estudos recentes mostram a presença de DNA do HPV em mais de 99,7% dos casos de neoplasia intraepitelial cervical (NIC), com taxas de expressão que variam de 40% a 70%, utilizando, por exemplo, a técnica da reação em cadeia da polimerase (PCR).[4]

Nas últimas décadas, o conhecimento da lenta história natural da evolução do câncer de colo uterino e da ampla utilização de métodos de rastreio para a detecção das lesões precursoras em fase inicial, tem demonstrado ser eficiente na prevenção secundária. Atualmente, a prevenção primária, através da utilização de vacinas contra o Papilomavírus Humano (HPV) tem sido testada [4,7-9] e serve como um dos melhores exemplos de que a doença pode ser prevenida.

De acordo com a Organização Mundial da Saúde de classificação de tumor cervical uterino, [10] as lesões precursoras do câncer de colo uterino são classificadas como NIC I, II e NIC III. Como existe uma dificuldade em diagnosticar tais precursores, eles também podem ser referidos como lesões de alto (NIC II e NIC III) e baixo grau (NIC I). Contudo, algumas vezes, em pequenas biópsias é muito difícil diferenciar as lesões de baixo e alto grau, e este diagnóstico é muito importante para o tratamento de um paciente.

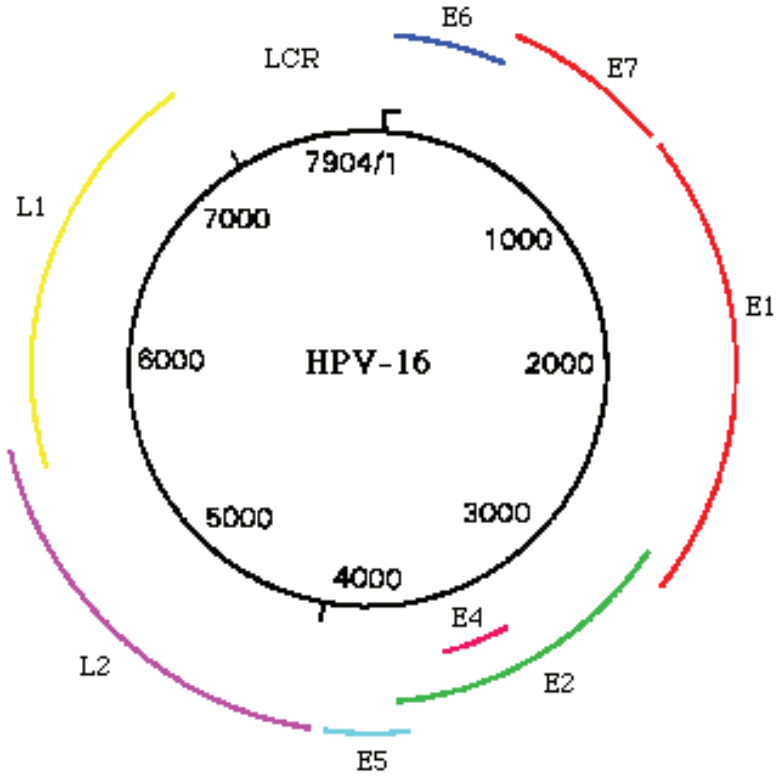
1.2 Papiloma Vírus Humano (HPV)

Está bem estabelecido que a infecção pelo HPV seja o principal fator causal de câncer cervical e sua infecção é transmitida através do contato sexual, sendo que os fatores de risco estão intimamente relacionados com o comportamento sexual (por exemplo, número de parceiros sexuais múltiplos e relação sexual em idade precoce).[3,4,6]

O HPV contém um DNA em dupla fita circular que mede cerca de 8.000 pares de bases no qual estão presentes as regiões gênicas E (*early*) e L (*late*). A região E está relacionada à replicação do DNA viral, controle de transcrição, maturação, alteração da matriz celular e estímulo de proliferação, além da transformação celular. Neste último fenômeno estão envolvidos os genes E6 e E7, que são os principais transformadores do HPV e estão diretamente envolvidos na indução de proliferação benigna e transformação maligna nas células do hospedeiro.[11] Geralmente o câncer cervical se desenvolve como conseqüência de alterações genéticas com inativação de genes supressores de tumor. Os mais estudados e que se relacionam com o potencial oncogênico do HPV têm sido os genes p53 e pRb. O papel dos produtos destes genes é regular o ciclo celular por controle da transcrição de genes celulares envolvidos na progressão do ciclo e na proliferação celular.[11]

As células do epitélio escamoso são alvos do HPV, capaz de provocar alterações que podem levar a um câncer cervical. Após a infecção, o vírus HPV se integra ao DNA do hospedeiro e se multiplicam em vários números de cópias.[7] Os genes E6 e E7 inativam o p53 e o pRb respectivamente, inibindo o mecanismo de controle celular da síntese de DNA. A malignização pode ocorrer pela instabilidade genética e mutações críticas dos oncogenes e estímulo de divisões celulares descontroladas, resultando em alterações grosseiras no código genético onde não podem ser reparadas, pois os

mecanismos utilizáveis para este fim estão bloqueados pela infecção do HPV.[7,11] As figuras 1 e 2 demonstram esquematicamente a estrutura interna e externa do Papilomavírus Humano.



Legenda: HPV – Papilomavírus Humano; E – região inicial que codifica oncoproteínas; L – região tardia que codifica proteínas do capsídeo; LCR – Longa região de controle.

Figura 1 – Organização do genoma do HPV 16, mostrando a localização dos genes.[12]

Figura 2 - Modelo anatômico do capsídeo do HPV [12]

1.2.1 Persistência do HPV

As mulheres que apresentam infecção persistente por tipos virais de alto risco do HPV são consideradas o verdadeiro grupo de risco para o desenvolvimento do câncer cervical. Estudos mostram que após o contágio, a freqüência da doença diminui progressivamente com a idade, sugerindo o desenvolvimento de uma imunidade específica. O grau de risco de desenvolver o câncer cervical está muito provavelmente relacionado à carga viral e à persistência da infecção.[2,5,13] Na maioria das mulheres, infecções pelo HPV são transitórias e não deixam seqüelas clínicas. Apenas cerca de dez por cento das mulheres persistem com a infecção por mais de seis meses dando origem a lesões de alto grau e que podem evoluir para carcinoma invasivo. [14,15]

1.3 Proteína p16

Vários estudos têm destacado o papel da proteína $p16^{INK4a}$ como um marcador de carcinoma cervical e que sua expressão está associada com a progressão da doença e diretamente relacionada com a presença de HPV. [4,7,16,17]

1.3.1 Proteína p16 como supressora tumoral

Dentro de condições normais, a proteína $p16^{INK4a}$ age como supressora tumoral inibindo as quinases dependentes de ciclina (CDK4 e CDK6), que regulam o ponto G1 de checagem do ciclo celular. A proteína nuclear pR interage com o fator de transcrição celular E2F na fase G1 do ciclo celular. Esta interação inibe a transcrição E2F induzida dos genes celulares envolvidos na proliferação e replicação de DNA. As ciclinas (CDK4 e CDK6) fosforilam a pRb, resultando na separação do complexo pRb-E2F. Esta separação de pRb permite a progressão do ciclo celular da fase G1 para S (vide figura 3).[18-22]

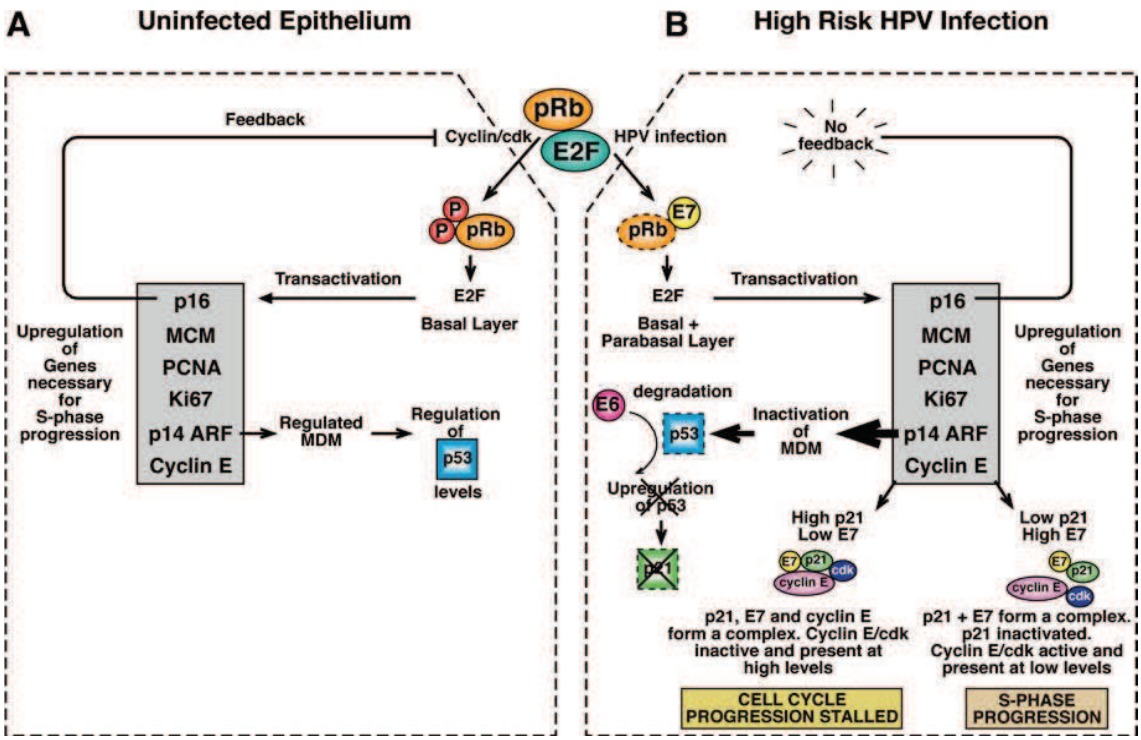
1.3.2 Proteína p16 e o HPV

A integração do HPV no DNA do hospedeiro acarreta alguns descontroles celulares. O gene E6 tem a função de inativar a proteína nuclear p53 resultando em uma instabilidade genética e mutações críticas. As proteínas do complexo pRb-E2F se separam na presença do gene E7, inativando a pRb e ativando o fator de transcrição celular E2F. Este, por sua vez, inicia a transcrição de genes necessários para a

replicação do DNA e, assim, impropriamente forçando passar a célula do ponto G1 / S para a fase S.[18-23] A inativação da pRb pelo gene E7 do HPV resulta na superexpressão de $p16^{INK4a}$, devido a um *feedback* negativo entre pRb e $p16^{INK4a}$ (vide figura 3).[18-22]

Segundo Branca et al (2004),[19] estas funções são características de E6 e E7 somente nos tipos de HPV de alto risco, pois em contraste, os genes E6 e E7 do HPV de baixo risco, são falhos em se ligar em p53 e pRb.

Em resumo, a expressão da proteína p16INK4a, pode ser detectada imunoistoquimicamente e está diretamente relacionada com a presença de HPV.[8,18,19] Assim, esta proteína pode ser usada como um biomarcador, adicionando uma significativa precisão diagnóstica na avaliação de lesões cervicais (NIC).[7,8,17]



Legenda: A- Epitélio não infectado. A molécula de pRb se separa do E2F através da fosforilação das ciclinas CDK4 e CDK6. A ativação do E2F leva à progressão do ciclo celular e a proteína p16 é ativada para inibir as ciclinas regulando o ciclo celular. B – Epitélio infectado por HPV de alto risco. O gene E7 se integra ao DNA do hospedeiro e inativa a molécula de pRb deixando o E2F livre para realizar a progressão do ciclo celular descontroladamente. A p16 é ativada para inibir esse descontrole celular

inativando as ciclinas, mas não obtêm uma resposta positiva. Em resposta, é observado uma grande produção e uma superexpressão dessa proteína nas células. [23]

Figura 3-Estimulação da progressão do ciclo celular, por tipos de HPV de alto risco.

1.4 Ki-67

O Ki-67 é um marcador de proteína não-histonica de proliferação celular, e é expressa em todas as fases do ciclo celular, exceto em G0.[24,25] Esta proteína tem a função de se expressar em crescimento de tumores humanos sugerindo o grau de malignidade.[26-28]

A interação de E6 e E7 de HPV no DNA da célula hospedeira perturba o ciclo celular acarretando em anormalidades na expressão de proteínas, incluindo a Ki-67.[18]

Alguns estudos têm mostrado que a positividade do Ki-67 na imunoistoquímica demonstra uma crescente proliferação de lesões intraepiteliais de baixo e alto grau.[29] Em outros, os resultados das análises são consistentes com uma forte relação entre p16 e Ki-67 no reconhecimento do HPV associada à lesões cervicais pré-neoplásicas.[18]

A proliferação descontrolada encontrada nas células malignas, marcadas pelo Ki-67, pode servir de diagnóstico para o câncer. Com isso, o Ki-67 parece ser um método promissor para o uso em patologia, por ter uma relação definida entre o ciclo celular, facilitando e permitindo análises relativamente simples e economicamente viáveis. [28]

1.5. Molécula de adesão E-caderina

As Caderinas são glicoproteínas de 120 a 130 kDa que estão envolvidas na adesão celular e recebem este nome por necessitarem de cálcio (Ca^{+2}), para se ligarem. A firme adesão intercelular atribuída à função de interações adesivas desempenhando um papel crucial na formação de tecidos e o seu envolvimento, consiste de um importante biomarcador para o desenvolvimento de tumores. (30-34)

As células escamosas do epitélio cervical são fortemente ligadas uma a outra e a membrana basal através de um grande número de moléculas de aderência. Assim, a E-caderina é uma das principais moléculas de aderência que definem a arquitetura do epitélio. Sabe-se que no câncer intra-epitelial cervical existe uma mudança na expressão dessa molécula. (31)

Isto sugere que a diminuição ou perda da expressão da E-caderina pode ser correlacionada com comportamento agressivo e progressão do câncer.(27) Roa et al. (2001),(35) consideram as Caderinas como os mais importantes mediadores de moléculas de adesão celular e mostrou que a perda desta molécula em tecidos de linhas tumorais está correlacionada com a capacidade de invadir o colágeno dos tecidos.

Presume-se a mutação das células reduz sua capacidade de aderirem umas às outras facilitando o seu desligamento em tumores primários e metástases. Assim, a diminuição na expressão da E-caderina parece ser um parâmetro útil na avaliação do potencial de malignidade de câncer cervical.(30)

Carico et al. (2001),(36) demonstraram uma correlação significativa entre a perda da E-caderina no epitélio exocervical de biópsias de mulheres infectadas pelo HPV sem lesões de maior gravidade, em relação as biópsias de lesões displásicas (de NIC I a NIC III) e carcinoma invasivo.

Dursun et al. (2007),[37] concluíram que a redução da expressão da E-caderina está associada significativamente com a sobrevida livre de doença em pacientes com carcinoma cervical, servindo como um indicador de comportamento clínico agressivo e poderia sugerir o uso da terapia adjuvante em estágios iniciais da doença.

1.6 Diagnóstico Histológico

Embora existam critérios histológicos para o diagnóstico de lesões cervicais, [38] muitas vezes o diagnóstico se torna mais vulnerável devido ao tamanho da amostra, e assim pode prejudicar a conduta clínica posterior. Como o custo do estudo imunoistoquímico é mais barato do que a técnica de PCR para análise da presença de positividade de HPV, seria interessante encontrar imunomarcadores para diferentes graus de lesão intraepitelial, principalmente para separar lesões de alto e baixo grau, devido ao diferente tratamento que é dado a cada um desses grupos.

1.7 Objetivos

1.7.1 Objetivo geral

Avaliar a expressão dos marcadores biológicos p16, Ki-67 e E-caderina em lesões de colo uterino.

1.7.2 Objetivos específicos

a. Estudar a expressão imunoistoquímica das proteínas p16, Ki-67 e E-caderina em lesões benignas, pré-invasivas e carcinoma invasivo de colo uterino.

b. Correlacionar a expressão destes marcadores juntos em casos de difícil interpretação, auxiliando no diagnóstico e prognóstico das lesões cervicais.

c. Avaliar a relação entre as expressão desses marcadores e a persistência ou não da lesão cervical.

2 ARTIGO

10 *The Open Pathology Journal*, 2009, *3*, 10-17

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 biomarkes don't differentiate between persistent CIN and no persistent lesions.

Keywords: p16, ki-67, E-cadherin, uterine cervical cancer, persistent cervical intracpithelial 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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1874-3757/09

Currently, primary prevention, through the use of vaccines against human Papillomavirus (HPV) has been demonstrated [4, 7-9].

According to the cervical uterine tumor classification of the World Health Organization (WHO, 2008) [10] the precursor lesions of cervical cancer are classified as CIN I, II and CIN III. Because there is a difficulty in diagnose such precursors, they can also be referred to as high and low grade lesions. However, sometimes in small biopsy is quite difficult to differentiate lesions from low to 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 and that p16 expression is associated with the progression of the disease and 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 *INK 4a.* 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 not passing to DNA replication. In cervical lesions induced by HPV, viral oncoprotein E7 interacts with pRb and inactivate it. As a result, the regulatory pathway Cdk-Rb-E2F is disrupted and inactivated pRb pass cell cycle checkpoint G1/S without any obstacle. As a response, a overexpression of p16 occurs. In turn, p16INK4a protein can be a marker of premalignant and malignant cervical epithelium cells. Functionally active gene *RB* was shown to be able to negatively regulate the expression of *INK4a* on a transcriptional level, but details of this negative feed-back loop remain obscure [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, 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 his marker could suggest the degree of malignancy [20-22].

The interaction of E6 and E7 HPV DNA from 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 use of Ki-67 immunohistochemistry positivity demonstrate the increasing proliferation in low and high grade 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 involve the cell adhesion receiving this name by needing 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 a tumor development [24-28].

The squamous cells of cervix epithelium are strongly attached to each other and 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.* (2001) [29] consider the Cadherins as the most important mediators of cell adhesion molecules and showed that the loss of this molecule in tumor tissues of lines determines the ability to invade the collagen of tissues.

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

Dursun *et al.* (2007) [30] concluded that reducing the expression of E-cadherin is significantly associated with overall survival and disease-free survival in 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 the uterine cervix and to correlate the expression of these markers together in cases with difficult interpretation, helping 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 cadherin-E (NCL-E, Novocastra, clone 36B5), for 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 4μm cuts were dew axed, undergone on antigenic recuperation, suffered peroxidase block and then were incubated into primary antibodies: ki-67 antigen mouse monoclonal antibody, dissolved 1 : 600, the cadherin-E mouse monoclonal antibody dissolved 1 : 200 and the p16 mouse monoclonal antibody dissolved 1 : 50. The incubation with the biotinilated anti-Ig antibody or secondary antibody was used which is specific for an animal species whose primary antibody was made (kit DAKO LSAB-labeled stretavidin biotin) for Ki-67 and cadherin-E; for p16, was used secondary antibody (kit NOVOLINK-Polymer Detection Systems), dissolved in PBS for 30 minutes at 37°C in a humid room. Next, stretavidin biotin peroxidase complex incubation was made (Kit, peroxidase-DakoCytomation, Carpinteria, CA, USA). For the revelation the cromogenic diaminobenzine subtract was used and were 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×. 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 one of a 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, 13of 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, p16, Ki-67 and E-cadherin positivity in different diagnosis.

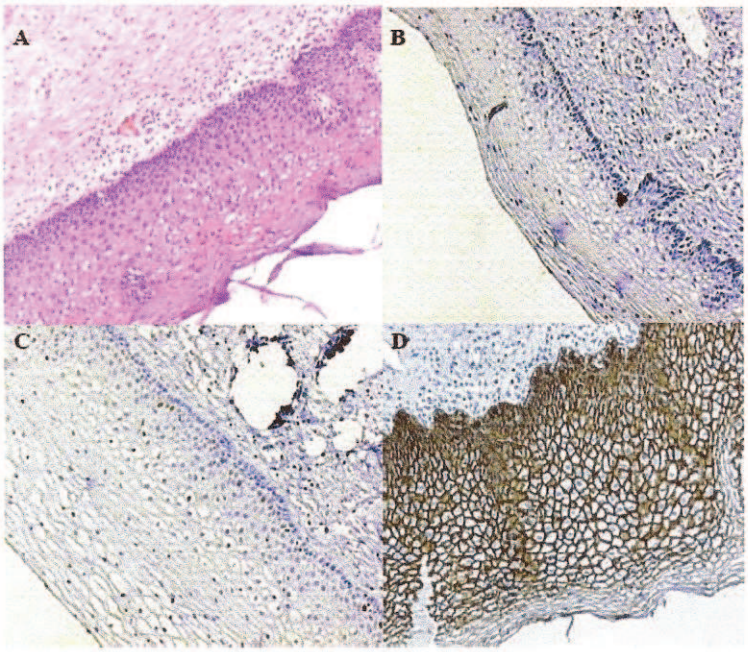

Fig. (1). Photomicrography of normal uterine cervix: (**A**) HE stain (100X); (**B**) p16 antibody (100X); (**C**) Ki-67 antibody (100X) and (**D**) E-cadherin antibody (100X). The number of positive cells for E-cadherin had the greatest expression (89.05%) when compared with the cervical lesions.. There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

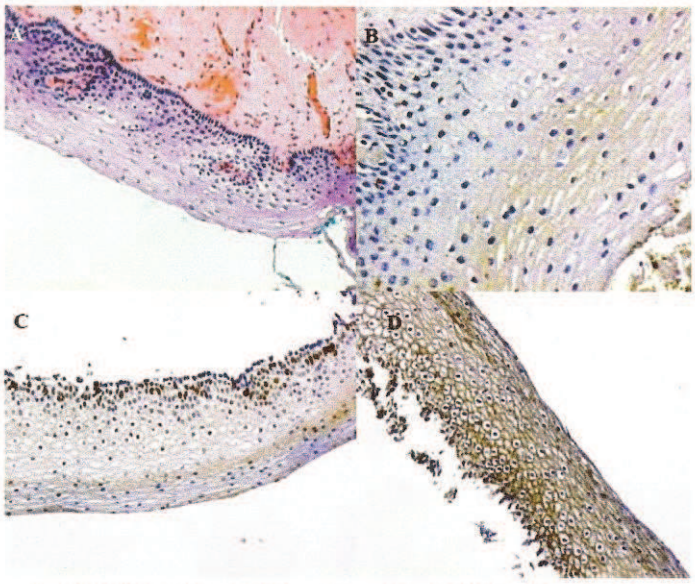

Fig. (2). Photomicrography of CIN I (Cervical intraepithelial neoplasia grade I): **(A)** HE stain (100X); **(B)** p16 antibody (200X); **(C)** Ki-67 antibody (100X) and **(D)** E-cadherin antibody (100X). The number of positive cells for p16 (27.94%) and Ki-67 (35.6%) was lower than for E-cadherin (82.18%).

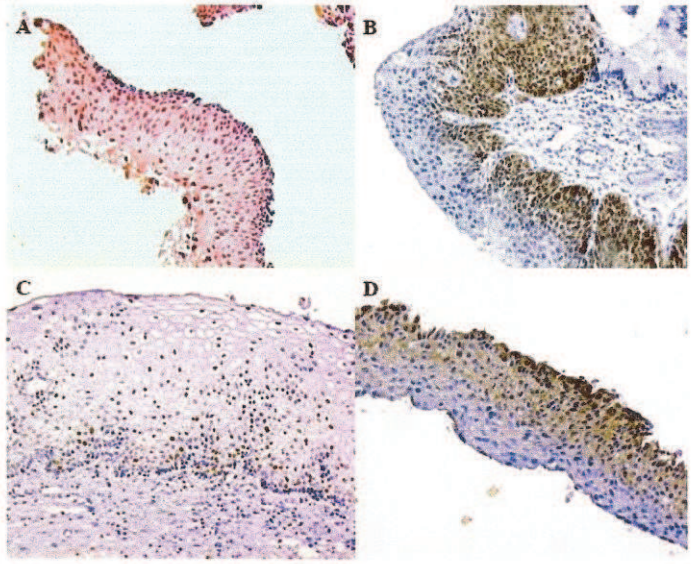

Fig. (3). Photomicrography of CIN II (Cervical intraepithelial neoplasia grade II): **(A)** HE stain (100X); **(B)** p16 antibody (100X); **(C)** Ki-67 antibody (100X) and **(D)** E-cadherin antibody (100X). The antibody E-cadherin had a higher expression (77.39%) when compared with p16 (47.93%) and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

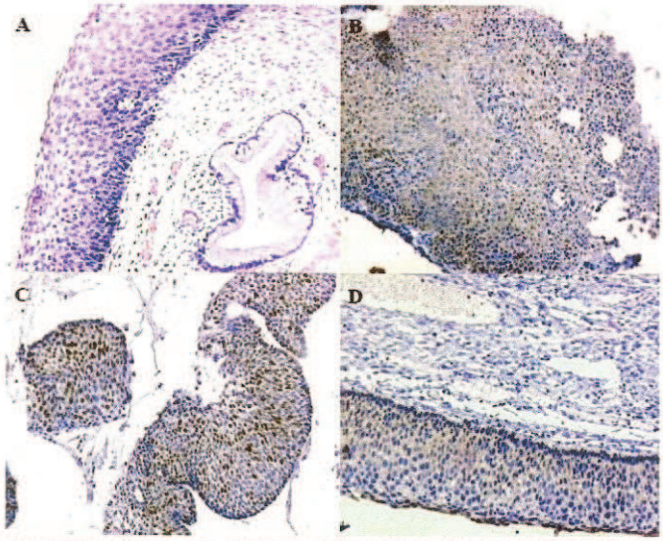

Fig. (4). Photomicrography of CIN III (Cervical intraepithelial neoplasia grade III): (**A**) HE stain (100X); (**B**) p16 antibody (100X); (**C**) Ki-67 antibody (100X) and (**D**) E-cadherin antibody (100X). The antibody p16 had a higher expression (73.47%) when compared with Ki-67 (40.9%) and the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

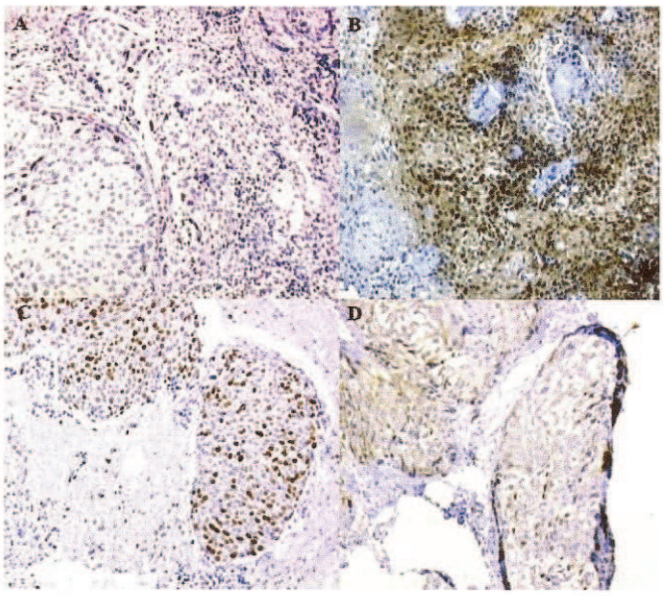

Fig. (5). Photomicrography of Invasive Carcinoma: (**A**) HE stain (200X); (**B**) p16 antibody (100X); (**C**) Ki-67 antibody (100X) and (**D**) E-cadherin antibody (100X). The number of positive cells for E-cadherin (46.15%) and Ki-67 (57.8%) was lower than for p16 antibody (72.70%).

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 in CIN III (73.47%) and invasive carcinoma (72.70%) ($p < 0.0005$).

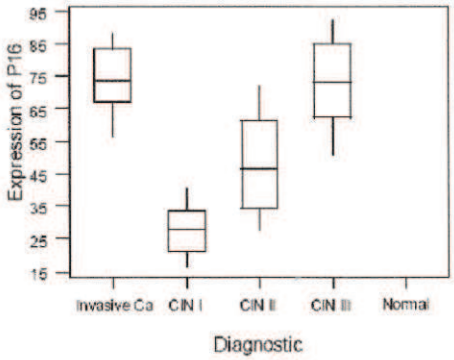

Fig. (6). Expression of p16 in different types of cervical lesion. There is an increase of p16 according to the degree of malignancy of injuries ($p < 0.0005$). Normal tissue did not present p16 expression. Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial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

Ki-67 Versus Diagnosis

Fig. (**7**) shows the different positivities to the Ki-67 in the groups evaluated. There was only statistically significant difference 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 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**).

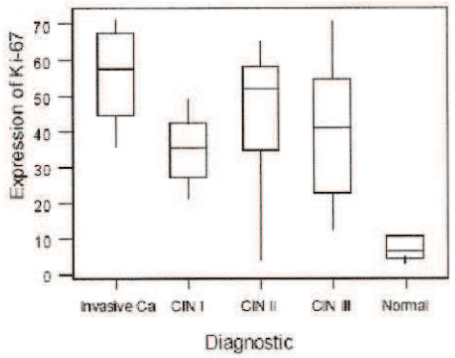

Fig. (7). Positive for Ki-67 in different types of injury of the uterine cervix. Ki-67 is higher expressed in neoplastic groups than in normal cervical biopsies ($p = 0.005$). Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN II - Cervical intraepithelial neoplasia grade II, CIN III - Cervical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

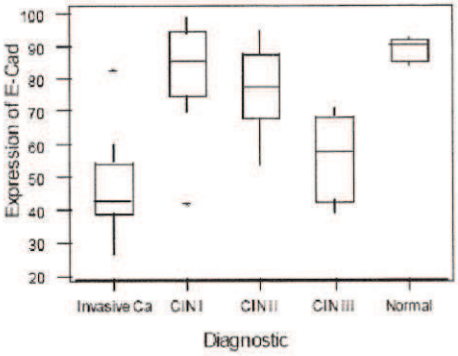

Fig. (8). Expression of E-cadherin in different types of injury of the uterine cervix. There was statistically significant difference when comparing the positive cases of invasive carcinoma (46.15%) with CIN I and CIN II ($p < 0.0005$). Legend: CIN I - Cervical intraepithelial neoplasia grade I, CIN 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 and may be a good marker to detect risk of developing cervical cancer in women infected by HPV.

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)

	BIOPSY			
HPV	**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.

All cases of SCC and CIN III in our study had an overexpression of p16. Similar evidence was obtained by Benevolo (2006) [11], Volgareva (2004) [31] and Tringler (2004) [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 (2006) [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.* 2004 [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 (2006) [12], Keating (2001) [13], Abeer (2007) [16], Volgereva (2004) [31], Tringler (2004) [32], Longato Filho (2005) [34] and Walts (2006) [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.

ACKNOWLEDGEMENT

We thank the financial support from CNPq and CAPES.

REFERENCES

[1] Franco EL. Epidemiologia do câncer mamário ginecológico. In.:Abrão F S. Tratado de Oncologia Genital e Mamária. São Paulo: Ed. Roca, 1995.

[2] Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Instituto Nacional de Câncer. Coordenação de Prevenção e Vigilância de Câncer. Estimativas 2008: Incidência de Câncer no Brasil. Rio de Janeiro: INCA, 2008. [Cited on Oct. 4]. Available from: http://www.inca.gov.br/estimativa/2008/versaofinal.pdf.

[3] Bauer HM, Hildesheim A, Schiffman MH, *et al.* Determination of genital Human papillomavirus infection in low risk womem in Portland, Oregon. Sex Transm Dis 1993; 20(5): 274-8.

[4] Queiroz C, Silva TC, Alves VAF, *et al.* P16(INK4a) expression as a potential prognostic marker in cervical pre- neoplastic and neoplastic lesions. Pathol Pract Res 2006; 202: 77- 83.

[5] Psyrri A, DiMaio D. The Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol 2008; 5(1): 24-31.

[6] Zampirolo JA, Merlin JC, Menezes ME. Prevalência de HPV de baixo e alto risco pela técnica de biologia molecular (Captura Híbrida II) em Santa Catarina. Rev Bras Anal Clin 2007; 39(4): 265-68.

[7] Von Knebel Doeberitz M. New markers of cervical dysplasia to visualc the genomic chaos created by aberrant oncogenic papillomavirus infections. Eur J Cancer 2002; 38(17): 2229-42.

[8] Soslow R, Isacson, C, Zaloudek C. Diagnostic Immunohistochemistry of the Female Genital Tract. In: Dabbs DJ (Ed). Diagnostic Immunohistochemistry. 2nd Ed. Churchill Livingstone: Philadelphia, 2006.

[9] INCA. Neoplasias Intra-epitelial Cervical - NIC. Rev Bras Cancerol 2005; 46(4): 355-57.

[10] World Health Organization. World Health Organization Classification of Tumours. Pathology & Genetics-Tumours of the Breast and Female Genital Organs. Lyon: IARC Scientific Publications, 2008 N. 4: 270.

[11] Guimarães MCM, Gonçalves MAG, Soares CP, Bettini JSR, Duarte RA, Soares EG. Immunohistochemical Expression of p16 and bcl-2 According to HPV Type and to the Progression of Cervical Squamous Intraepithelial Lesions. J Histochem Cytochem 2005; 53(4): 509-16.

[12] Benevolo M, Mottolese M, Marandino F, *et al.* Immunohistochemical expression of p16(INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. Mod Pathol 2006; 19(3): 384-91.

[13] Keating JT, Cviko A, Riethdorf S, *et al.* Ki-67, cyclin E, and p16INK4 are complementary surrogate biomarkers for human papillomavirus-related cervical neoplasia. Am J Surg Pathol 2001; 25(7): 884-91.

[14] Branca M, Ciotti M, Santini D, *et al.* P16 (INK4A) expression is related to grade of cin and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. Int J Gynecol Pathol 2004; 23(4): 354-65.

[15] renna SMF. Expressão proteica de P53 e C-MYC como marcadores no prognostico do carcinoma de colo uterino. [tese]. Campinas: Universidade Estadual de Campinas. Faculdade de Ciências Medicas, 2000.

[16] Bahnassy AA, Zekri ARN, Saleh M, Lotayef M, Moneir M, Shawki O. The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma. BMC Clin Pathol 2007; 7: 4.

[17] Howley PM, Lowy DR. Papillomaviruses and their replication. In: Knipe DM, Howley PM (Ed.). Fields Virology, 4th Ed., Lippincott Williams & Wilkins: Philadelphia, 2001. pp 2198-29.

[18] Prowse DM, Ktori EN, Chandrasekaran D, Prapa A, Baithun S. Human papillomavirus-associated increase in p16INK4A expression in penile lichen sclerosus and squamous cell carcinoma. Br J Dermatol 2008; 158(2): 261-5.

[19] Cotran RS, Kumar V, Abbas AK, Fausto N, Robbins e Cotran Patologia-Bases Patológicas das Doenças. 7ª Ed. Rio de Janeiro: Elsevier, 2005.

[20] Kruse AJ, Baak JPA, Jansen EA, *et al.* Ki-67 predicts progression in early CIN: Validation of a Multivariate progression-risk model. Cell Oncol 2004; 26(1-2): 13-20.

[21] Harris TG, Kulasingam SL, Kiviat NB, *et al.* Cigarette smoking, oncogenic homan papilomavirus, Ki-67 antigen, and cervical intraepithelial neoplasia. Am J Epidemiol 2004; 159(9): 834-42.

[22] Cambruzzi E, Zettler CG, Alexandre COP. Expression of Ki-67 and Squamous Intraepithelial Lesions are related with HPV in Endocervical Adenocarcinoma. Pathol Oncol Res 2005; 11(2): 114-20.

[23] Isacson C, Theodore DK, Hedrick L, Cho KR. Both Cell Proliferation and Apoptosis Increase with Lesion Grade in Cervical Neoplasia but do not correlate with Human Papillomavirus Type. Cancer Res 1996; 56(2): 669-74.

[24] Kaplanis K, Kiziridou A, Liberis V, Destouni Z, Galazios G. E-cadherin expression during progression of squamous intraepithelial lesions in the uterine cervix. Eur J Gynaecol Oncol 2005; 26(6): 608-10.

[25] Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis and prognosis. Lung Cancer 2002; 36(2): 115-24.

[26] Yaldizl M, Hakverdi AU, Bayhan G, Akkuş Z. Expression of E-cadherin in squamous cell carcinomas of the cervix with correlations to clinicopathological features. Eur J Gynaecol Oncol 2005; 26(1): 95-8.

[27] Rosenau J, Bahar MJ, Wasiellewski R, *et al.* Ki-67, E-cadherin, and p53 as prognostic indicators of long-term outcome after liver transplation for metastatic neuroendocrine tumors. Transplantation 2002; 73(3): 386-94.

[28] Van de Putte G, Kristensen GB, Baekelandt M, Lie AK, Holm R. E-cadherin and Catenins in early squamous cervical carcinoma. Gynecol Oncol 2004; 94(2): 521-7.

[29] Roa IE, Villaseca M, Araya JC, Roa J, Aretxabala XU, Miranda M. Moléculas de adhesión celular y cancer. Rev Chil Cir 2001; 53(5): 504-510.

[30] Dursun P, Yuce K, Usubutun A, Ayhan A. Loss of epithelium cadherin expression is associated with reduced overall survival and disease-free survival in early-stage squamous cell cervical carcinoma. Int J Gynecol Cancer 2007; 17(4): 843-50.

[31] 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.

[32] Tringler B, Gup CJ, Singh M, *et al.* Evaluation of p16INK4A and pRb expression in cervical squamous and glandular neoplasia. Hum Pathol 2004 ;35(6): 689-96.

[33] Maehama T. Epidemiological study in Okinawa, Japan, of human papilomavirus infection of the uterine cervix. Infect Dis Obstet Gynecol 2005; 13(2): 77-80.

[34] Longato Filho A, Utagawa ML, Shiarata KN, *et al.* Immunocytochemical expression of p16INK4A and Ki-67 in cytologically negative and equivocal pap smears positive for oncogenic human papillomavirus. Int J Gynecol Pathol 2005; 24(2): 118-24.

[35] Walts AE, Lechago J, Bose S. P16 and Ki67 Immunostaining is a Useful Adjunct in the Assessment of Biopsies for HPV-Associated Anal Intraepithelial Neoplasia. Am J Surg Pathol 2006; 30(7): 795-801.

[36] Vinh-Hung V, Bourgain C, Vlastos G, *et al.* Prognostic value of histopathology and trends in cervical cancer: a SEER population study. BMC Cancer 2007; 7: 164.

[37] Jemal A, Siegel R, Ward E, *et al.* Cancer statistics, 2008. CA Cancer J Clin 2008; 58(2): 71-96.

[38] Jain RV, Mills PK, Parikh-Patel A. Cancer incidence in the south Asian population of California. 1988-2000. J Carcinog 2005; 4: 21.

[39] Parkin DM, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. Eur J Cancer 2001; 37(Suppl 8): S4-66.

[40] Rodriguez AC, Burk R, Herrero R, *et al.* The natural history of human papillomavirus infection and cervical intraepithelial neoplasia among young women in the Guanacaste cohort shortly after initiation of sexual life. Sex Transm Dis 2007; 34(7): 494-502.

[41] Kahn JA, Lan D, Kahn RS. Sociodemographic Factors Associated With High-Risk Human Papillomavirus Infection. Obstet Gynecol 2007; 110(1): 87-95.

[42] Nakagawa, M, Kim KH, Gillam TM, Moscicki AB. HLA Class I Binding Promiscuity of the CD8 T-Cell Epitopes of Human Papillomavirus Type 16 E6 Protein. J Virol 2007; 81(3): 1412-23.

[43] Meijer CJ. Detection of HPV in cervical scrapes by PCR in relation to cytology: Possible implications for cancer screening. In: Munoz N, Bosh FX, Shan KV (Ed.). The epidemiology of HPV and cervical cancer. Lyon: IARC Scientific Publications, 1992, pp. 271-81.

Received: January 1, 2009 Revised: January 12, 2009 Accepted: January 19, 2009

3 CONCLUSÕES

Pela análise dos resultados, podemos concluir que:

1 a – Em relação à proteína p16 vimos que houve um aumento de sua expressão de acordo com o aumento do grau das lesões. Em tecidos normais não houve expressão da mesma.

1 b – Para o Ki-67, observou-se uma maior expressão dessa proteína nos casos de carcinoma invasivo em relação ao epitélio normal. Porém, não houve diferença significativa de sua expressão quando comparamos o carcinoma invasivo com NIC I, II e III.

1 c – Observou- se expressão mais acentuada de E-caderina em tecidos normais de colo uterino e em NIC I. Entretanto, existe diferença estatatística apenas quando comparada a expressão de carcinoma invasivo com NIC I e NIC II.

2 - A associação dos marcadores p16, Ki-67 e E-caderina, nos casos de difícil interpretação anátomo-patológica, podem ajudar no diagnóstico de lesões cervicais para distinguir os casos com provável evolução para carcinoma espinocelular.

3 - O uso dos biomarcadores não são úteis para diferenciar entre NIC persistentes e não-persistente.

REFERÊCIAS BIBLIOGRÁFICAS

1 Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Instituto Nacional de Câncer. Coordenação de Prevenção e Vigilância de Câncer. Estimativas 2008: Incidência de Câncer no Brasil. Rio de Janeiro: INCA, 2007. 94p.: il. color.; tab http://www.inca.gov.br/estimativa/2008/versaofinal.pdf. Acessado Outubro 4, 2008.

2 Gompel C. Rastreamento do Câncer do Colo Uterino. 1ª ed. São Paulo: Andrei; 2007. p. 139-46.

3 Bauer HM, Hildesheim A, Schiffman MH, Glass AG, Rush BB, Scott DR, et al. Determination of genital Human papillomavirus infection in low risk womem in Portland, Oregon. Sex Transm Dis 1993;20(5):274-8..

4 Queiroz C, Silva TC, Alves VAF, Villa LL, Costa MC, Travassos AG, et al. P16(INK4a) expression as a potential prognostic marker in cervical pre- neoplastic and neoplastic lesions. Pathol Pract Res 2006;202: 77- 83.

5 Psyrri A, DiMaio D. The Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol. 2008;5(1):24-31.

6 Zampirolo JA, Merlin JC, Menezes ME. Prevalência de HPV de baixo e alto risco pela técnica de biologia molecular (Captura Hibrida II) em Santa Catarina . Rev Bras Anal Clin 2007;39(4):265-268.

7 von Knebel Doeberitz M. New markers of cervical dysplasia to visuale the genomic chaos created by aberrant oncogenic papillomavirus infections. Eur J Cancer 2002;38(17):2229-42.

8 Soslow R, Isacson, C, Zaloudek C. Diagnostic Immunohistochemistry of the Female Genital Tract. In: Dabbs DJ (Ed). Diagnostic Immunohistochemistry. 2nd ed. Churchill Livingstone: Philadelphia; 2006.

9 INCA. Neoplasias Intra-epitelial Cervical - NIC. Rev Bras Cancerol 2005;46(4):355-57.

10 World Health Organization. World Health Organization Classification of Tumours. Pathology & Genetics – Tumours of the Breast and Female Genital Organs. Lyon: IARC Scientific Publications; 2008. N. 4: 270.

11 Junior JE; Giraldo PC; Gonçalves AK. Immunohistochemistry markers for lesions preceding HPV-induced cervical cancer:The role of tumoral suppression p16ink4a protein. DST – J Bras Doenças Sex Transm 2006; 18(1):62-65.

12 HPV – Papilomavírus Humano. In: Papovaviruses. Disponivel em: www.mcb.uct.ac.za/cann/335/Papovaviruses.html. Acessado Janeiro 7, 2009.

13 Kajaer SK, van den Brule AJC, Svare EI, Engholm G, Sherman ME, Poll PA. Different risk factor patterns for high-grade and low-grade Intraepithelial lesions on the cervix among HPV-positive and HPV-negative young women. Int J Cancer; 1998; 76**:** 613–619.

14 Melkert PW, Hopman E, van den Brule AJ, Risse EK, van Diest PJ, Bleker OP, et al. Prevalence of HPV in cytomorphologically normal cervical smears, as determined by the polymerase chain reaction, is age-dependent. Int J Cancer; 1993;53(6):919-23.

15 Remmink AJ, Walboomers JM, Helmerhorst TJ, Voorhorst FJ, Rozendaal L, Risse EK, et al. The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. Int J Cancer 1995;61(3):306-11.

16 Guimarães MCM, Gonçalves MAG, Soares CP, Bettini JSR, Duarte RA, Soares EG. Immunohistochemical Expression of p16 and bcl-2 According to HPV Type and to the Progression of Cervical Squamous Intraepithelial Lesions. J Histochem Cytochem 2005;53(4):509-16.

17 Benevolo M, Mottolese M, Marandino F, Vocaturo G, Sindico R, Piperno G, et al. Immunohistochemical expression of p16(INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. Mod Pathol 2006;19(3):384-91.

18 Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, et al. Ki-67, cyclin E, and p16INK4 are complementary surrogate biomarkers for human papillomavirus-related cervical neoplasia. Am J Surg Pathol 2001;25(7):884-91.

19 Branca M, Ciotti M, Santini D, Di Bonito L, Giorgi C, Benedetto A, et al. P16 (INK4A) expression is related to grade of cin and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. Int J Gynecol Pathol 2004;23(4):354-65.

20 Brenna SMF. Expressão proteica de P53 e C-MYC como marcadores no prognostico do carcinoma de colo uterino. [tese]. Campinas: Universidade Estadual de Campinas. Faculdade de Ciências Medicas; 2000.

21 Bahnassy AA, Zekri ARN, Saleh M, Lotayef M, Moneir M, Shawki O. The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma. BMC Clin Pathol 2007; 7:4.

22 Howley PM, Lowy DR. Papillomaviruses and their replication. In: Knipe DM, Howley PM (Ed.). Fields Virology, 4th edition. Lippincott Williams & Wilkins: Philadelphia; 2001. p. 2198-229.

23 Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Scien 2006; 110: 525 – 541.

24 Prowse DM, Ktori EN, Chandrasekaran D, Prapa A, Baithun S. Human papillomavirus-associated increase in p16INK4A expression in penile lichen sclerosus and squamous cell carcinoma. Br J Dermatol 2008;158(2):261-5.

25 Cotran RS, Kumar V, Abbas AK, Fausto N. Robbins e Cotran Patologia – Bases Patológicas das Doenças. 7ª ed. Rio de Janeiro: Elsevier; 2005.

26 Kruse AJ, Baak JPA, Jansen EA, Kjellevold KH, Fiane B, Lovslett K, et al. Ki-67 predicts progression in early CIN: Validation of a Multivariate progression-risk model. Cell Oncol 2004;26(1-2):13-20.

27 Harris TG, Kulasingam SL, Kiviat NB, Mao C, Agoff SN, Feng Q, et al. Cigarette smoking, oncogenic homan papilomavirus, Ki-67 antigen, and cervical intraepithelial neoplasia. Am J Epidemiol 2004;159(9):834-42.

28 Cambruzzi E, Zettler CG, Alexandre COP. Expression of Ki-67 and Squamous Intraepithelial Lesions are related with HPV in Endocervical Adenocarcinoma. Pathol Oncol Res 2005;11(2):114-20.

29 Isacson C, Theodore DK, Hedrick L, Cho KR. Both Cell Proliferation and Apoptosis Increase with Lesion Grade in Cervical Neoplasia but do not correlate with Human Papillomavirus Type. Cancer Res 1996; 56(2):669-674.

30 Kaplanis K, Kiziridou A, Liberis V, Destouni Z, Galazios G. E-cadherin expression during progression of squamous intraepithelial lesions in the uterine cervix. Eur J Gynaecol Oncol 2005;26(6):608-10.

31 Bremnes RM, Veve R, Hirsch FR, Franklin WA. The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis and prognosis. Lung Cancer 2002;36(2):115-24.

32 Yaldizl M, Hakverdi AU, Bayhan G, Akkuş Z. Expression of E-cadherin in squamous cell carcinomas of the cervix with correlations to clinicopathological features. Eur J Gynaecol Oncol 2005;26(1):95-8.

33 Rosenau J, Bahar MJ, Wasiellewski R, Mengel M, Schimidt HHJ, Nashan B, et al. Ki-67, E-cadherin, and p53 as prognostic indicators of long-term outcome after liver transplation for metastatic neuroendocrine tumors. Transplantation 2002;73(3):386-94.

34 van de Putte G, Kristensen GB, Baekelandt M, Lie AK, Holm R. E-cadherin and Catenins in early squamous cervical carcinoma. Gynecol Oncol 2004 Aug;94(2):521-7.

35 Roa IE, Villaseca M, Araya JC, Roa J, Aretxabala XU, Miranda M. Moléculas de adhesión celular y cancer . Rev Chil Cir 2001;53(5):504-510.

36 Carico E, Atlante M, Bucci B, Nofroni I, Vecchione A. E-cadherin and α-Catenin Expression during Tumor pregressión of cervical carcinoma. Gynecol Oncol 2001;80:156-61.

37 Dursun P, Yuce K, Usubutun A, Ayhan A. Loss of epithelium cadherin expression is associated with reduced overall survival and disease-free survival in early-stage squamous cell cervical carcinoma. Int J Gynecol Cancer 2007;17(4):843-50.

38 Volgareva G, Zavalishina L, Andreeva Y, Frank G, Krutikova E, Golovina D, et al. Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. BMC Cancer 2004;4:58.

Apêndices

Quadro 1 – Expressão imunoistoquímica dos marcadores nos casos de colo uterino sem alterações

Paciente	Diagnóstico	Persistente	Não- persistente	índice p16	índice Ki-67	índice E-cad
50	Normal			*	11%	86%
51	Normal			*	11%	92%
52	Normal			*	6%	84%
53	Normal			*	7%	90%
54	Normal			*	3%	92%

Quadro 2 – Expressão imunoistoquímica dos marcadores nos casos de Neoplasia intra-epitelial cervical grau I (NIC I)

Paciente	Diagnóstico	Persistente	Não-persistente	índice p16	índice Ki-67	índice E-cad
1	NIC I	X		28%	26%	74%
2	NIC I	X		*	41%	77%
3	NIC I	X		*	31%	91%
4	NIC I	X		40%	38%	96%
5	NIC I		X	17%	24%	80%
6	NIC I		X	28%	49%	76%
7	NIC I		X	32%	36%	70%
8	NIC I		X	*	44%	94%
9	NIC I		X	*	32%	93%
10	NIC I	X		23%	21%	42%
11	NIC I		X	*	42%	94%

Quadro 3 - Expressão imunoistoquímica dos marcadores nos casos de Neoplasia intra-epitelial cervical grau II (NIC II)

Paciente	Diagnóstico	Persistente	Não- persistente	índice p16	índice Ki-67	índice E-cad
13	NIC II	X		*	57%	88%
14	NIC II	X		*	65%	95%
15	NIC II	X		*	59%	53%
16	NIC II	X		29%	45%	80%
17	NIC II		X	72%	33%	72%
18	NIC II		X	40%	40%	75%
19	NIC II		X	55%	64%	67%
20	NIC II	X		68%	52%	68%
21	NIC II		X	28%	21%	88%
22	NIC II		X	45%	56%	83%
23	NIC II		X	49%	4%	92%
24	NIC II	X		46%	52%	68%

Quadro 4 - Expressão imunoistoquímica dos marcadores nos casos de Neoplasia intra-epitelial cervical grau III (NIC III) ou carcinoma *in situ*

Paciente	Diagnóstico	Persistente	Não- persistente	índice p16	índice Ki-67	índice E-cad
25	NIC III		X	89%	52%	39%
26	NIC III		X	73%	12%	42%
27	NIC III		X	88%	37%	69%
28	NIC III		X	92%	28%	66%
29	NIC III	X		72%	59%	47%
30	NIC III		X	61%	58%	50%
31	NIC III		X	51%	21%	41%
32	NIC III		X	80%	21%	46%
33	NIC III		X	64%	41%	*
34	NIC III		X	82%	25%	65%
35	NIC III		X	69%	50%	71%
36	NIC III		X	78%	41%	66%
37	NIC III		X	56%	71%	70%

Quadro 5 - Expressão imunoistoquímica dos marcadores nos casos de carcinoma invasivo do colo uterino

Paciente	Diagnóstico	Persistente	Não- persistente	índice p16	índice Ki-67	índice E-cad
38	Ca Invasivo		X	80%	69%	32%
39	Ca Invasivo		X	86%	64%	83%
40	Ca Invasivo		X	57%	61%	26%
41	Ca Invasivo		X	69%	48%	38%
42	Ca Invasivo		X	65%	40%	40%
43	Ca Invasivo		X	75%	71%	43%
44	Ca Invasivo		X	69%	36%	43%
45	Ca Invasivo		X	75%	55%	60%
46	Ca Invasivo		X	73%	71%	42%
47	Ca Invasivo		X	84%	43%	57%
48	Ca Invasivo		X	67%	54%	44%
49	Ca Invasivo		X	88%	62%	46%

Legenda: * (casos que apresentaram negatividade na expressão do marcador ou que foi perdido o material).