

# Immunohistochemical Expression of the Tumor Suppressor Protein p16<sup>INK4a</sup> in Cervical Adenocarcinoma

## *Expressão imunohistoquímica da proteína de supressão tumoral p16<sup>INK4a</sup> em adenocarcinoma cervical*

José Eleutério Jr<sup>1</sup> Thiago Silva Lima<sup>1</sup> Maria do Perpétuo Socorro Cunha<sup>2</sup>  
Diane Isabelle Magno Cavalcante<sup>1</sup> Angélica Maria Holanda Silva<sup>1</sup>

<sup>1</sup> Department of Pathology, Faculty of Medicine, Universidade Federal do Ceará (UFC), Fortaleza, CE, Brazil

<sup>2</sup> Department of Surgical Pathology, Instituto do Câncer do Ceará, Fortaleza, CE, Brazil

**Address for correspondence** José Eleutério Jr., Department of Pathology, Faculdade de Medicina, Universidade Federal do Ceará (UFC), Alexandre Baraúna, 94,- Rodolfo Teófilo, 60430-160 Fortaleza, Ceará, Brazil (e-mail: prof.eleuterio@gmail.com).

Rev Bras Ginecol Obstet 2017;39:21–25.

### Abstract

**Objective** To evaluate the diagnostic utility of the p16<sup>ink4a</sup> protein expression as a marker for adenocarcinoma of the cervix.

**Methods** In a cross-sectional study, p16<sup>ink4a</sup> expression was evaluated in 30 cervical biopsies from patients diagnosed with invasive adenocarcinoma from 2 reference clinics in Brazil, and compared with 18 biopsies of endocervical polyps (control cases). The performance of the tests for p16<sup>ink4a</sup> was evaluated using a conventional contingency table, and the Kappa ( $\kappa$ ) index was used to evaluate the agreement of the marker with the tissue diagnosis.

**Results** In total, 66% of the invasive adenocarcinoma cases were positive for p16<sup>ink4a</sup>. All of the adenomatous polyps cases used as negative controls were shown to be negative for p16<sup>ink4a</sup>. The marker showed a high sensitivity and a high negative predictive value. The Kappa index was good for p16<sup>ink4a</sup> ( $\kappa = 0.6$ ).

**Conclusion** Considering the strong association between the p16<sup>ink4a</sup> marker and the cervical adenocarcinoma, its use represents an important tool for reducing incorrect diagnoses of adenocarcinoma and thereby avoiding overtreatment.

### Keywords

- ▶ p16<sup>INK4a</sup>
- ▶ adenocarcinoma
- ▶ cervix
- ▶ immunohistochemistry

### Resumo

**Objetivo** Avaliar a utilidade diagnóstica da expressão da proteína p16<sup>ink4a</sup> como marcador de adenocarcinoma do colo.

**Métodos** Em estudo transversal, a expressão de p16<sup>ink4a</sup> foi avaliada em 30 biópsias cervicais de pacientes diagnosticadas com adenocarcinoma invasivo de colo uterino provenientes de dois serviços de referência no Brasil, comparando com achados em 18 biópsias de pólipos endocervicais (grupo de controle). Para avaliar a performance do

**Palavras-chave**

- ▶ p16<sup>INK4a</sup>
- ▶ adenocarcinoma
- ▶ colo uterino
- ▶ imunoistoquímica

teste, foi utilizada tabela de contingência convencional, e para avaliar a concordância com o diagnóstico, foi aplicado o índice de Kappa ( $\kappa$ ).

**Resultados** No total, 66% dos casos de adenocarcinoma invasivo foram positivos para p16<sup>ink4a</sup>. Todos os pólipos adenomatosos foram negativos para p16<sup>ink4a</sup>. O marcador mostrou uma alta sensibilidade e alto valor preditivo negativo. O índice de Kappa foi bom para p16<sup>ink4a</sup> ( $\kappa = 0.6$ ).

**Conclusion** Considerando a forte associação entre o marcador p16<sup>ink4a</sup> e o adenocarcinoma cervical, seu uso representa uma ferramenta importante para reduzir o risco de diagnóstico incorreto de adenocarcinoma e, por conseguinte, evitar o excesso de tratamentos.

**Introduction**

Cervical cancer is an important and frequent cause of death in women worldwide. It is estimated that ~ 500,000 new cases occur annually. Approximately 80% of the reported cases are located in less developed countries.<sup>1</sup> In Brazil, according to the National Cancer Institute (INCA), cervical cancer is the third most common cancer overall, and the fourth leading cause of cancer death in women.<sup>2</sup>

Squamous cell carcinoma is the most common histological type of cervical cancer, representing 80–90% of cases. Adenocarcinoma is an uncommon type, but the incidence of adenocarcinoma has been increasing over the last two decades, especially in women under 40 years of age.<sup>3</sup> This type of neoplasia presents in various distinct histological patterns.<sup>4</sup> According to the World Health Organization (WHO), the pathological types of cervical adenocarcinoma include the following: mucinous (endocervical, intestinal, ring signet), endometrioid, clear cell, serous and mesonephric. The most prevalent types are mucinous and endometrioid carcinoma, which together comprise ~ 90% of all cases.<sup>5</sup>

The risk factors for this type of neoplasia are: the human papillomavirus (HPV), oral contraceptives, high parity, smoking and sexual behavior.<sup>4</sup> The HPV is a DNA virus that can infect the anogenital region. Approximately 18 types are oncogenic (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 63, 66, 68, and 82) and associated with squamous cell carcinoma and adenocarcinoma.<sup>6,7</sup>

The HPV oncoproteins E6 and E7 interact with tumor suppressor proteins involved in the cell cycle, such as p53, and with those associated with proliferation, such as the retinoblastoma protein (pRb). When the latter protein interacts with E7, cyclin-dependent kinase (CDK) complexes are activated, resulting in unrestricted and abnormal cell proliferation; furthermore, p53 bound to E6 cannot promote the apoptosis of these abnormal cells. These oncoproteins degrade p53 and pRb, and stimulate (via negative feedback) the exaggerated expression of the p16<sup>ink4a</sup> protein.<sup>6,7</sup>

The *p16* (*CDKN2/INK4a*) gene, classified as a tumor suppressor, negatively controls cell cycle progression at the G1/S checkpoint.<sup>8–10</sup> The *p16* gene inhibits the group of regulatory

proteins called CDKs, which activate or inhibit specific phases of the cell cycle.<sup>8</sup>

Thereby, the expression of the p16 protein indicates the possibility of a pre-invasive or invasive lesion.<sup>9</sup> In addition, this protein has shown potential to discriminate cervical adenocarcinoma from endometrial adenocarcinoma.<sup>10–12</sup>

The p16 protein has been the focus of several studies that demonstrated that its overexpression can be applied as a marker of HPV-induced cervical lesions, as well as a predictor of poor prognosis.<sup>13–15</sup> These studies mainly involved squamous cell carcinoma, but a few studies have shown results regarding the expression of p16<sup>ink4a</sup> in cases of cervical adenocarcinoma and its association with the various histological types.

**Methods**

This cross-sectional study analyzed cervical biopsies from the surgical pathology services files of the Pathology Department of Universidade Federal do Ceará and of Instituto do Câncer do Ceará. In order to be included in the study, the blocks had to contain sufficient material to prepare slides for histopathology and immunohistochemistry.

Paraffin blocks of tissue were processed into 4- $\mu$ m sections, and the sections were stained with hematoxylin-eosin (HE) for morphological diagnosis; the concordance of a double-blind evaluation by two independent pathologists was required to ensure the diagnoses of adenocarcinomas. Cases with either dissimilar diagnosis or with unsatisfactory material for evaluation were excluded from the study. In total, 30 cases met the inclusion criteria.

The staining for p16<sup>ink4a</sup> was performed using the CINtec® histology kit (MTM Laboratories, Heidelberg, Germany) on 4- $\mu$ m sections of formalin-fixed, paraffin-embedded specimens on slides with 10% poly-L-lysine (Sigma-Aldrich, St. Louis, Missouri, US). As a final step, the slides received a light hematoxylin counterstain. Endocervical polyp biopsies (18 samples) were used as the control group (negative cases for p16<sup>ink4a</sup>) for comparison.

The evaluation of p16<sup>ink4a</sup> expression was performed as described by Schorge et al<sup>16</sup>, using scores for the intensity and the percentage of positive tumor cells (nuclear and cytoplasmic stain). The following scores were used to

evaluate the intensity of p16 expression: 0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining. The following scores were used to evaluate the percentage of p16-positive cells: 0: none; 1: 5%; 2: 6–25%; 3: 26–50%; 4: 51–75%; 5: > 75%. The staining intensity scores were added to the percentage of positive tumor cell scores to obtain a total expression score that could range from 0–8, with 0 indicating no expression, and 8 indicating maximum marker expression.

The performance of the tests for p16<sup>ink4a</sup> in detecting cervical adenocarcinoma lesions was evaluated using conventional contingency tables to calculate the sensitivity, the specificity, and the positive and negative predictive values. The Kappa ( $\kappa$ ) index was used to determine the agreement of the markers with the tissue diagnosis.

The research project was approved by the Ethics Committee in Research of Universidade Federal do Ceará (Protocol number: 042/2011).

## Results

The expression of p16<sup>ink4a</sup> was evaluated in 30 cases of cervical adenocarcinoma. Among the 30 cases of invasive adenocarcinoma: 19 (63.3%) were endocervical adenocarcinomas; 3 (10%) were clear cell adenocarcinomas; 1 (3.3%) was a poorly differentiated solid adenocarcinoma; 1 (3.3%) was a serous adenocarcinoma; 1 (3.3%) was an endocervical adenocarcinoma associated with a squamous cell carcinoma in situ; 1 (3.3%) was an endometrioid adenocarcinoma with a villoglandular component; 1 (3.3%) was an adenosquamous carcinoma; and 1 (3.3%) was a minimal deviation adenocarcinoma.

**Table 1** p16<sup>ink4a</sup> expression in cases diagnosed with cervical adenocarcinoma and adenomatous polyps

Histopathology	p16 <sup>ink4a</sup> negative	p16 <sup>ink4a</sup> positive
Endocervical polyp	18 (100%)	0 (0%)
Invasive adenocarcinoma	10 (33.4%)	20 (66.6%)

All 18 cases (100%) of endocervical polyps were negative for p16<sup>ink4a</sup>, whereas 66% (20/30) of the cases of invasive adenocarcinoma were positive for p16<sup>ink4a</sup> (► **Table 1**). Twenty of the 30 cervical adenocarcinoma cases (66.7%) demonstrated strong expression for p16<sup>ink4a</sup>. Considering only the cases of endocervical adenocarcinoma, 12/19 (63%) exhibited strong or moderate expression. The expression of p16<sup>ink4a</sup> in other types of adenocarcinoma is demonstrated in ► **Table 2** (► **Fig. 1**).

The expression of p16<sup>ink4a</sup> demonstrated a sensitivity of 66.67% and a specificity of 100%, and the positive and negative predictive values were 100% and 64.29% respectively. The Kappa index, which assesses the diagnostic agreement between the histopathology and the expression of p16<sup>ink4a</sup>, was 0.60 (good agreement) (► **Table 3**).

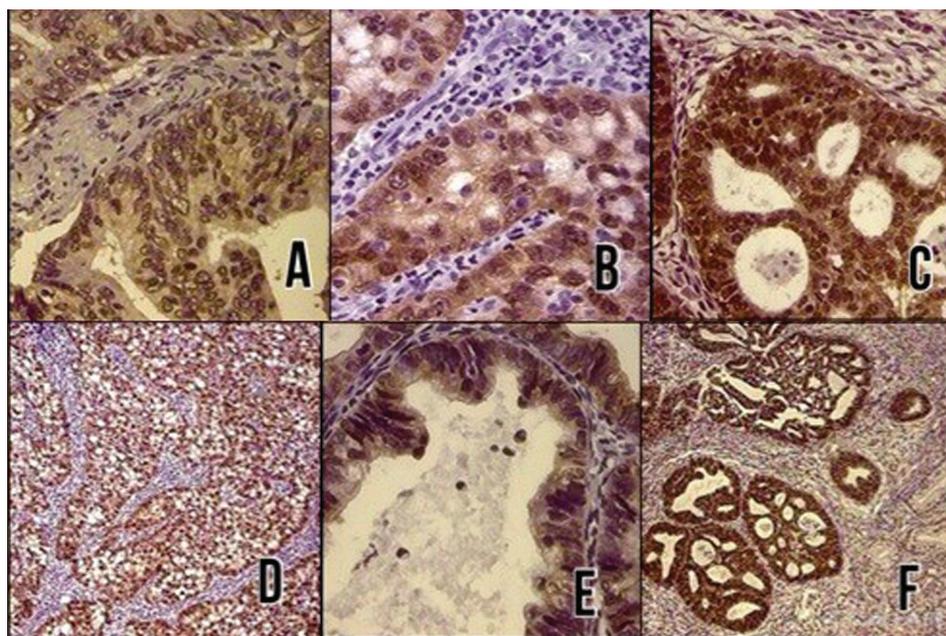
## Discussion

The variability in the histological patterns of cervical adenocarcinomas associated with glandular reaction phenomena can lead to diagnostic pitfalls, suggesting the need for biomarkers that can be used to correctly diagnose the lesions.

**Table 2** Expression of the p16<sup>ink4a</sup> marker in cells from the biopsies of patients diagnosed with endocervical adenomatous polyps or adenocarcinoma of the cervix

Histopathology	p16 <sup>ink4a</sup> negative	p16 <sup>ink4a</sup> positive	Total
Endocervical polyp	18 (100%)	0 (0%)	18(100%)
Endocervical ADC	7 (36.8%)	12 (63.2%)	19(100%)
Endometrioid ADC	0 (0%)	3 (100%)	3(100%)
Poorly differentiated ADC	0 (0%)	1 (100%)	1(100%)
Serous ADC	0 (0%)	1 (100%)	1(100%)
ADC with a villoglandular component	0 (0%)	1 (100%)	1(100%)
Endocervical ADC + CIS	0 (0%)	1 (100%)	1(100%)
Adenosquamous	1 (100%)	0 (0%)	1(100%)
Clear cell ADC	1 (50%)	1 (50%)	2(100%)
Minimal deviation ADC	1 (100%)	0 (0%)	1(100%)
Total	28 (50%)	20 (50%)	48 (100%)

Abbreviations: ADC, adenocarcinoma; CIS, epidermoid carcinoma in situ.



**Fig. 1** p16<sup>ink4a</sup> immune staining in biopsies of cervical adenocarcinoma in which the scores of the antibody expression vary from 6 (E – 100x) and 7 (A – 400x) to 8 (B – 400x, C – 400x, D – 100x, F – 100x). Picture D is of a clear cell adenocarcinoma.

Among the biomarkers for cervical neoplasia, p16<sup>ink4a</sup> has been the subject of several studies.<sup>12–18</sup>

The 30 cases of invasive adenocarcinoma evaluated in this study included 68% mucinous adenocarcinomas, 6.6% clear cell adenocarcinomas, and other types, with 3% each. These findings are consistent with a previous study.<sup>19</sup>

The distinction between endocervical adenocarcinoma and reactive pictures is not always easy. Abu-Backer et al<sup>10</sup> considered p16<sup>ink4a</sup> to be an excellent marker for distinguishing benign endocervical glandular lesions from malignant ones. This study corroborated this previous finding: all cases of adenomatous polyps showed no expression of p16<sup>ink4a</sup>, whereas most of the cervical adenocarcinoma cases were positive (66%). However, Li et al<sup>20</sup> and Riethdorf et al<sup>21</sup> detected positive expression of p16<sup>ink4a</sup> in tubal metaplasia and normal endocervical gland cells. According to Anghebem-Oliveira and Merlin,<sup>22</sup> rare foci of p16<sup>ink4a</sup> positivity in a small proportion of normal and metaplastic squamous epithelium might be observed because, under physiological conditions such as genomic stress, p16 is expressed.

Yonamine et al<sup>23</sup> noted that the frequency of positive p16<sup>ink4a</sup> expression in adenocarcinomas of the cervix was 80%. However, they studied cases of both in situ and invasive

cervical adenocarcinoma. In our study, which only evaluated invasive adenocarcinoma, the p16<sup>ink4a</sup> expression occurred in 66% of the cases. The absence of expression might be explained by the lack of an association between the histological type and the HPV. Thus, although the expression is highly suggestive of a tumoral lesion, the lack of expression, which is expected in normal cases, can occur in some cases of adenocarcinoma.

In this study, the performance of p16<sup>ink4a</sup> in diagnosing cervical adenocarcinomas had a high sensitivity (66%), high specificity (100%) and high negative and positive predictive values (64% and 100% respectively). The diagnostic agreement index was good. Li et al<sup>21</sup> reported that the over-expression of p16<sup>ink4a</sup> had a sensitivity of 100% as a marker for detecting cervical adenocarcinoma in situ.

The diagnostic performance of the marker can be evaluated for the diagnosis of adenocarcinoma, considering the gold standard agreement between two pathologists blindly and taking into account the observed Kappa that was considered good. From this, we can infer that, although not absolute in the final diagnosis, doubtful cases can be clarified with the use of p16<sup>ink4a</sup>.

The assessment performed on adenocarcinoma cases has some limitations because it is a study of file blocks; the process of fixation might compromise the immunohistochemical expression. Furthermore, human papillomavirus

**Table 3** Sensitivity, specificity, and negative and positive predictive values for p16<sup>ink4a</sup> in the diagnosis of adenocarcinomas of the cervix

Immunohistochemical marker	Sensitivity	Specificity	PPV	NPV	Kappa (CI)	SE
p16 <sup>ink4a</sup>	66.7%	100%	100%	64.29%	0.6 (0.398–0.802)	0.103

Abbreviations: CI, confidence interval (95%); NPV, negative predictive value; PPV, positive predictive value; SE, standard error. Note: Kappa: < 0, very bad; 0–0.2, bad; 0.21–0.4, reasonable; 0.41–0.6, good; 0.61–0.8, very good; 0.81–1, excellent.

analysis was not performed, and only a small number of cases were included due to their low incidence in the clinical setting.

In conclusion, considering the association between the p16<sup>INK4a</sup> and endocervical carcinomas, the use of p16<sup>INK4a</sup> represents an important tool for reducing incorrect diagnoses of adenocarcinoma. Even though a high specificity of p16<sup>INK4a</sup> was demonstrated, there are cases of invasive adenocarcinomas that do not express it. Studies that include a larger number of cases and HPV genotyping should be encouraged to clarify the remaining controversial issues.

#### Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

#### References

- Capote Negrin LG. Epidemiology of cervical cancer in Latin America. *Ecancelmedscience* 2015;9:577
- Brasil. Ministério da Saúde. Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA) [Internet]. [Estimate 2012: incidence of cancer in Brazil]. Rio de Janeiro: INCA; 2011 [cited 2016 Mar 15]. Available from: [http://portal.saude.sp.gov.br/recursos/ses/perfil/gestor/homepage/estimativas-de-incidencia-de-cancer-2012/estimativas\\_incidencia\\_cancer\\_2012.pdf](http://portal.saude.sp.gov.br/recursos/ses/perfil/gestor/homepage/estimativas-de-incidencia-de-cancer-2012/estimativas_incidencia_cancer_2012.pdf). Portuguese.
- Dahlström LA, Ylitalo N, Sundström K, et al. Prospective study of human papillomavirus and risk of cervical adenocarcinoma. *Int J Cancer* 2010;127(08):1923–1930
- Silverberg SG, Ioffe OB. Pathology of cervical cancer. *Cancer J* 2003;9(05):335–347
- Young RH, Clement PB. Endocervical adenocarcinoma and its variants: their morphology and differential diagnosis. *Histopathology* 2002;41(03):185–207
- Termini L, Villa LL. [Biomarkers in cervical cancer screening]. *J Bras Doenças Sex Transm* 2008;20(02):125–131 Portuguese
- Rosa MI, Medeiros LR, Rosa DD, Bozzeti MC, Silva FR, Silva BR. [Human papillomavirus and cervical neoplasia]. *Cad Saude Publica* 2009;25(05):953–964 Portuguese.
- McCluggage WG. Immunohistochemical and functional biomarkers of value in female genital tract lesions. *Int J Gynecol Pathol* 2006;25(02):101–120
- Cameron RI, Maxwell P, Jenkins D, McCluggage WG. Immunohistochemical staining with MIB1, bcl2 and p16 assists in the distinction of cervical glandular intraepithelial neoplasia from tubo-endometrial metaplasia, endometriosis and microglandular hyperplasia. *Histopathology* 2002;41(04):313–321
- Abu Backer FM, Nik Mustapha NR, Othman NH. Clinico-pathological comparison of adenocarcinoma of cervix and endometrium using cell cycle markers: P16<sup>INK4a</sup>, P21<sup>waf1</sup>, and p27<sup>Kip1</sup> on 132 cancers. *Infect Dis Obstet Gynecol* 2011; 2011:857851
- Wang JL, Zheng BY, Li XD, Angström T, Lindström MS, Wallin KL. Predictive significance of the alterations of p16<sup>INK4a</sup>, p14<sup>ARF</sup>, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer. *Clin Cancer Res* 2004;10(07): 2407–2414
- Yanaranop M, Ayuwat S, Nakrangsee S. Differential diagnosis between primary endocervical and endometrial adenocarcinoma using immunohistochemical staining of estrogen receptor, vimentin, carcinoembryonic antigen and p16. *J Med Assoc Thai* 2016;99(Suppl 2):S106–S115
- Eleutério J Jr, Giraldo PC, Gonçalves AKS, et al. Prognostic markers of high-grade squamous intraepithelial lesions: the role of p16<sup>INK4a</sup> and high-risk human papillomavirus. *Acta Obstet Gynecol Scand* 2007;86(01):94–98
- Kong CS, Balzer BL, Troxell ML, Patterson BK, Longacre TA. p16<sup>INK4a</sup> immunohistochemistry is superior to HPV in situ hybridization for the detection of high-risk HPV in atypical squamous metaplasia. *Am J Surg Pathol* 2007;31(01):33–43
- Pecorelli S, Zigliani L, Odicino F. Revised FIGO staging for carcinoma of the cervix. *Int J Gynaecol Obstet* 2009;105(02):107–108
- Schorge JO, Lea JS, Elias KJ, et al. P16 as a molecular biomarker of cervical adenocarcinoma. *Am J Obstet Gynecol* 2004;190(03): 668–673
- Caponio MA, Addati T, Popescu O, et al. P16(INK4a) protein expression in endocervical, endometrial and metastatic adenocarcinomas of extra-uterine origin: diagnostic and clinical considerations. *Cancer Biomark* 2014;14(02/03):169–175
- Pinto AP, Degen M, Villa LL, Cibas ES. Immunomarkers in gynecologic cytology: the search for the ideal 'bimolecular Papanicolaou test'. *Acta Cytol* 2012;56(02):109–121
- Alfsen GC, Thoresen SO, Kristensen GB, Skovlund E, Abeler VM. Histopathologic subtyping of cervical adenocarcinoma reveals increasing incidence rates of endometrioid tumors in all age groups: a population based study with review of all nonsquamous cervical carcinomas in Norway from 1966 to 1970, 1976 to 1980, and 1986 to 1990. *Cancer* 2000;89(06):1291–1299
- Li C, Rock KL, Woda BA, Jiang Z, Fraire AE, Dresser K. IMP3 is a novel biomarker for adenocarcinoma in situ of the uterine cervix: an immunohistochemical study in comparison with p16(INK4a) expression. *Mod Pathol* 2007;20(02):242–247
- Riethdorf L, Riethdorf S, Lee KR, Cviko A, Löning T, Crum CP. Human papillomaviruses, expression of p16, and early endocervical glandular neoplasia. *Hum Pathol* 2002;33(09):899–904
- Anghebem-Oliveira MI, Merlin JC. [Is the p16 protein a new marker for neoplastic progression in the cervix?] *Rev Bras Anal Clín.* 2010;42(03):181–185 Portuguese.
- Yonamine PTK, Junqueira MSG, Rodrigues JO, et al. [Association between p16 expression and cervical intraepithelial neoplasia]. *Arq Ciênc Saúde.* 2009;16(04):161–165 Portuguese.