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ARTICLE

Expression of Ki-67 and Squamous Intraepithelial Lesions are Related with HPV in Endocervical Adenocarcinoma

Eduardo CAMBRUZZI, Cláudio Galleano ZETTLER, Cláudio Osmar Pereira ALEXANDRE

Fundação Faculdade Federal de Ciências Médicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

To estimate the association between human papillomavirus (HPV) status and the expression of p53, Ki-67 and bcl-2 in cases of endocervical adenocarcinoma, and the relation with squamous intraepithelial lesions (SIL) and age, 229 cases were selected, treated between 1995 and 2003 in the Hospital Nossa Senhora da Conceição. All samples were evaluated by polymerase chain reaction to determine HPV status. Immunohistochemical technique was used to investigate the expression of p53, Ki-67 and bcl-2. The joint occurrence of endocervical adenocarcinoma and SIL were estimated too. In the 229 evaluated cases, 182 cases (79.48%) were associated with the presence of the HPV. The most common types were HPV18 (93 cases – 51.09%) and HPV16 (62 cases – 34.06%). Expression of Ki-67 (p=0.009) and the presence of SIL (p=0.018) were associated to HPV infection. Expression of p53 (p=0.647) and bcl-2 (p=0.671) were not related to HPV status. The mean age of the patients was 53.2 years, without clear correlation between age group and HPV (p=0.095). The presence of HPV, especially type 18 in endocervical adenocarcinoma suggests that this agent can be an important cofactor in the development and progression of glandular neoplasms of the uterine cervix. The joint occurrence of endocervical adenocarcinoma and SIL may support this hypothesis. HPV may promote an increased proliferation index in endocervical adenocarcinoma, shown by the expression of Ki-67. (Pathology Oncology Research Vol 11, No 2, 114–120)

Key words: endocervical adenocarcinoma, bcl-2, Ki-67, p53, HPV

Introduction

The endocervical adenocarcinoma accounts for 15 to 20% of the primary neoplasms of the uterine cervix, and its incidence has been increasing in the last years. Malignant neoplasms of the uterine cervix are preferentially located in the squamous-columnar junction, and the most common histological types are squamous carcinoma and adenocarcinoma. In general, adenocarcinoma presents a more aggressive biological behavior and worse prognosis.[1-3] Histogenetically, endocervical adenocarcinoma evolves from endocervical glands or even from reserve cells at the level of the glandular-squamous transition. The endocervical adenocarcinoma is a heterogeneous group of neoplasms, with a wide variability of architectural patterns. Besides the mucinous subtype, which represents most of these cases, there are the endometrioid, serous, villoglandular, mesonephric, clear cell-, and minimal deviation subtypes.[4-7]

Evidences suggest a close relation between HPV and cervical carcinogenesis. The HPV types of high oncogenic risk are associated with SIL and squamous carcinoma. Etiologically, HPV18 is more frequently associated with adenocarcinoma than with squamous carcinoma, suggesting a certain tropism of this type for glandular cells. HPV infection may be associated with changes in the function or expression of certain genes or proteins of the host's cells, such as p53 and bcl-2. The detection of these alterations may be a relevant parameter in the diagnosis and association with HPV.[8-12]

Apoptosis is essential for cell and tissue physiology, as well as cell division and differentiation. Apoptosis and cell proliferation are consistently related, the same mechanisms and phases concurring during the cell cycle. Most of the cells progress to phase G1 of the cell cycle, which is regulated by specific proteins like p53. Proteins E6 and

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Correspondence: Eduardo CAMBRUZZI, MD, Av. Loureiro da Silva, 1500/1308, B. Centro CEP: 90050-240, Porto Alegre, Rio Grande do Sul, Brazil; Tel.: 55-51-32261779 or 55-51-33782800, E-mail: dudacambruzzi@yahoo.com.br

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E7 of HPV types 16 and 18 may bind avidly to p53, thus inactivating its protective effect against abnormal proliferation of anomalous cells, and favoring neoplastic cell growth. Inactivation of p53 is considered one of the most important routes for the malignant transformation of cervical epithelium. P53 may even induce apoptosis, as it enables the formation of pro-apoptotic agents, such as bax, and inhibits apoptosis-repressive agents such as bcl-2.[13-17] The protein bcl-2 is situated mainly in the internal mitochondrial membrane, and is related to anti-apoptotic events and cell survival. Apoptosis in pre-cancerous lesions seems to reflect a physiological effort to eliminate genetically altered cells. Given that inhibition of apoptosis by bcl-2 can induce a genetic instability during the process of DNA repair, thus permitting the accumulation of genetic alterations and progression to malignant neoplasms, it is suggested that this protein is also involved in the malignant transformation of cells.[13,18-21]

Proliferation index of a neoplasm can be estimated by the immunohistochemical expression of Ki-67. In the normal cervix, Ki-67 expression is restricted to the basal and parabasal layers of the ectocervix, the metaplastic epithelium and the basal layer of the endocervix. Variations in the expression of this antigen can be found in the atrophic epithelium and during the different stages of the menstrual cycle. In the development of intraepithelial lesions, expression of Ki-67 can be found in the intermediate and surface layers of the epithelium, and the greater the number of positive cells and the farther they are from the basal membrane, the greater the grade of lesion. In cervical intraepithelial lesions associated with infections by HPV types 16 and 18, the expression of Ki-67 is greater than in lesions unrelated to viral presence.[12,22-25] By evaluating benign endocervical lesions, glandular intraepithelial dysplasias and cases of adenocarcinoma of the cervix, Heatley estimated a difference in the immunohistochemical expression of Ki-67 of up to 40% between these pathological conditions.[26]

If integration of HPV16 and -18 DNA into the genome of host cells determines the immortalization of these cell groups, inducing the malignant transformation of this epithelium and reflecting specific alterations in the cell cycle, it is possible that the cases of endocervical adenocarcinoma associated with HPV infection show increased rates of cell proliferation, inhibition of apoptosis, and anomalous expression of p53 as compared to cases not associated with the virus. Thus, the cases of endocervical adenocarcinoma which show co-expression of p53, bcl-2 and Ki-67 would be associated with the presence of HPV, corroborating data of the link between this virus and cell cycle proteins and the development of endocervical adenocarcinoma.[9,10,14,15]

The aim of this study was to estimate the association between the presence of HPV and immunohistochemical expression of p53, Ki-67 and bcl-2 in cases of endocervical adenocarcinoma. The data obtained were also related to the age group and the joint occurrence of squamous intraepithelial lesions.

Materials and methods

Patients group

All the cases of primary invasive adenocarcinoma of the uterine cervix evaluated from 1995 to 2003 at the Department of Pathology and Cytopathology of Grupo Hospitalar Conceição, in Porto Alegre, Brazil, were selected. The study period encompassed 94 months, and the study's ethical concepts were approved by the above mentioned institution. The cases of endocervical adenocarcinoma comprised 229 patients, initially evaluated by the Department of Gynecology of the same institution. Based on clinical investigation and histological analysis, all cases classified as epidermoid carcinoma, adenosquamous carcinoma, stromal neoplasm, benign epithelial neoplasm, other histological types of carcinoma except for adenocarcinoma, and metastatic carcinomas were excluded from the study.

For each specimen, 3-micron-thick histological sections were obtained to prepare five distinct slides for light microscopy, one of these for hematoxylin-eosin staining and the others, previously silanized, for the immunohistochemical study.

The specimens stained with hematoxylin-eosin were evaluated by two pathologists, individually and in collaboration, in order to confirm the diagnosis of primary invasive endocervical adenocarcinoma. There was a 100% concordance between the previous diagnosis and the data obtained by the study (Kappa test ratio = +1). The histological criteria determined by the WHO were strictly followed in determining primary adenocarcinoma of the uterine cervix. The samples analyzed comprised 109 usual biopsies of the cervix, 36 samples of cold-knife conization, 6 of high frequency conization, 39 products of cervical canal curettage, 16 pieces of total hysterectomy (with bilateral adnexectomy), and 23 of panhysterectomy (Wertheim-Meigs).

Immunohistochemistry

For immunohistochemical staining, sections were deparaffinized with xylene and hydrated with ethanol. Antigen recovery was performed in a microwave oven with a 10 mM/pH 6.0 citric acid solution in two cycles of 9 min each at 750 W. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Primary antibodies were diluted in 1% albumin solution and 0.1% sodium azide in PBS, applied in a humid chamber for 30 min at 37°C, and subsequently at 4°C for 18 h. The biotinylated secondary antibody was employed in a humid chamber at 37ºC for 30

min, followed by incubation with streptavidin-biotin-peroxidase complex (StreptABC). The chromogenic substrate used was 60 mg% diaminobenzidine in PBS, and Harrys hematoxylin was used for counterstaining. The antibodies used were monoclonal anti-p53, clone DO-7 (DAKO Corporation), monoclonal anti-Ki-67, clone MIB-1 (DAKO Corporation), monoclonal anti-human bcl-2, clone bcl-2/100/D5 (NOVOCASTRA Laboratories), and polyclonal anti-papillomavirus, BPV-1 (DAKO Corporation). The immunohistochemical expression in each case was estimated by the percentage of cells demonstrating specific staining with the antibody used. The following quantification scale was used: negative (0% of neoplastic cells showing antigen expression); mild positive (antigen expression present in 1-10% of neoplastic cells); moderate positive (antigen expression present in 11-50% of neoplastic cells); intense positive (antigen expression present in 51% or more of neoplastic cells).

Polymerase chain reaction

All the cases of adenocarcinoma were evaluated by PCR in order to determine the presence of the HPV genome in the neoplastic cells. From each case (paraffin blocks), areas containing only neoplastic glandular cells were selected by microscopic dissection, in order to study only the tumoral tissue. Ten-micron-thick sections were obtained from each sample, and placed in Eppendorf tubes. The specimens were submitted to deparaffinization with xylene and 96% ethanol, enzymatic digestion using proteinase K, and guanidine isothiocyanate extraction. The PCR technique employed primers GP5+/GP6+, which amplify a 140-bp fragment of the HPV in the L1 region. For each reaction 2 µl of the solution containing the sample's DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 3 µl of $MgCl_2$, 100 mM dATP, dGTP and dTTP, 2 µl of each primer, and 0.2 µl of Taq DNA polymerase were used, in a final volume of 50 µl. The amplifications were performed in a thermocycler, with a program of 40 cycles including 3 min at 94°C, 1 min at 94°C, 2 min at 40°C, and 1.5 min at 72°C. Products were evaluated in 1.5% agarose gel containing 5 µl of ethidium bromide. Specific primers for the sequence of oligonucleotides of proteins E6 and E7 were used for evaluation of HPV types 16 and 18.

Statistical analysis

The Kappa test was used for the statistical analysis to confirm the histological diagnosis of primary invasive endocervical adenocarcinoma. Non-parametric Mann-Whitney's test was selected as statistical test to analyze the association between the presence of HPV and immunohistochemical antigen expression. Spearman's test was used to evaluate the correlation between the immunohistochemical markers. The chi-square test was used to evaluate the relation between age groups and the presence of HPV, and to estimate the association between endocervical adenocarcinoma and squamous intraepithelial lesions.

Table 1. **Immunohistochemical expression of p53, bcl-2 and Ki-67 in cases of endocervical adenocarcinoma**

Antigen (N=229)	*Immunohistochemical reaction; n (%)*			
	Negative	*Mild*	*Moderate*	*Intense*
p53	107 (46.72)	36 (15.32)	74 (32.31)	12 (5.24)
bcl-2	145 (63.32)	28 (12.23)	42 (18.34)	14 (6.11)
Ki-67	22 (9.61)	32 (13.97)	117 (51.09)	58 (25.32)

Results

Molecular analysis and association between the presence of HPV and age groups

In the review of 229 cases, minimum and maximum ages were 21 and 94 years, with a median age of 54.5 years and mean age of 53.2 years. In age group distribution, 3 patients (1.3%) were between 21 and 30 years, 32 (13.9%) between 31 and 40 years, 51 (22.3%) between 41 and 50 years, 71 (31.1%) between 51 and 60 years, 40 (17.5%) between 61 and 70 years, 22 (9.6%) between 71 and 80 years, 9 (3.9%) between 81 and 90 years, and one patient (0.4%) between 91 and 100 years of age.

Among these 229 cases of adenocarcinoma, the PCR technique determined the presence of HPV in 182 cases (79.48%), 93 cases associated with HPV18 (51.09%), 62 cases associated with HPV16 (34.06%), and other 27 specimens (14.85%) associated with other HPV types.

The mean age of patients with HPV associated with adenocarcinoma (n=182) was 50.8 years. The HPV-negative patients (n=47) had a mean age of 54.7 years. Univariate analysis of the results relating the presence of HPV to patient age did not show statistically significant association (p=0.095).

Immunohistochemical expression of p53, bcl-2 and Ki-67, and correlation between antigen expressions

The immunohistochemical investigation demonstrated the expression of p53, bcl-2 and Ki-67 as shown in *Table 1*. The expression of p53 and Ki-67 was considered positive when a clear immunoreactivity was observed in the nucleus. Positive immunohistochemical expression of bcl-2 was found in the cytoplasm of neoplastic cells. The immunohistochemical analysis using the investigated antibodies determined positive reaction with p53 in 122 cases (53.27%), Ki-67 in 207 samples (90.39%), and bcl-2 in 84 cases (36.68%). The statistical correlation between the three markers did not prove to be significant (*Table 2*).

Table 2. **Association of immunohistochemical expression of p53, bcl-2 and Ki-67, and correlation with the presence of HPV**

Antigen (N=229)	*Antigen expression** *Mean*	SD	*HPV status (p)*	*bcl-2*	*p53*	*Ki-67*
bcl-2	12.9	16.1	0.671	–	0.629	0.259
p53	15.6	19.4	0.647	0.629	–	0.433
Ki-67	31.2	22.5	0.009	0.259	0.433	–

*% of positive cells in samples

Association of the presence of HPV and expression of p53, bcl-2 and Ki-67

Data in *Table 2* indicate a significant association between the presence of HPV and immunohistochemical expression of Ki-67 (p=0.009), with no relationship between the presence of HPV and expression of p53 and bcl-2. The expression of the immunohistochemical markers was also assessed as the joint association of two markers with the presence of HPV, but the results obtained did not indicate a statistically significant correlation, again highlighting the association of HPV with immunopositivity for Ki-67 (*Table 3*).

Associated squamous intraepithelial lesions

The joint occurrence of endocervical adenocarcinoma and squamous intraepithelial lesions was observed in 72 cases (31.5%), and it was found to be associated with HPV status (p=0.018). Among these intraepithelial lesions, 9 (12.5%) were low-grade (NIC I) and 63 (87.5%) high-grade (with 8 cases classified as NIC II and 55 as NIC III). Also, there were 3 (1.3%) associated cases of focal epidermoid carcinoma.

Discussion

Endocervical adenocarcinoma has come to play a major clinical and pathological role in the last two decades, probably because of a real and relative increase in its prevalence. Glandular lesions of the uterine cervix, similarly to squamous carcinoma and SIL, are associated with HPV. The presence of HPV can be demonstrated by PCR in 20-90% of the cases of endocervical adenocarcinoma. Other agents, such as the use of oral contraceptives, can be related to the development of this neoplasia. The mean age of patients affected by adenocarcinoma is about 50 years.[8,12,27,28] In this series, the mean age was 53.2 years. Although no significant relation between the age group of the patients and the presence of HPV was found in our study, viral infection is generally more frequent in young women, because they are more sexually active, and have not yet developed an adequate immune response against the infectious agent, which is present in older patients.[29-32]

Table 3. **Correlation between immunohistochemical expression of p53, bcl-2 and Ki-67**

Antigens	*HPV+*	*HPV–*
Bcl-2 – p53	0.724	0.260
Ki-67 – p53	0.271	0.672
Ki-67 – bcl-2	0.180	0.766

In the present study, 182 cases (79.48%) among 229 samples were associated with the presence of HPV, 93 (51.09%) with type 18 and 62 (34.06%) with type 16. Stanczuk et al. found the presence of HPV in 98 cases of carcinoma of the uterine cervix, of which 95 were squamous carcinomas and 3 adenocarcinomas. HPV DNA was detected in 97% of the samples, the most common ones being types 16 (61%), 33 (39%), 18 (18%) and 31 (4%). All adenocarcinoma cases were associated with HPV18.[33] Nakagawa et al. observed that 94 (98.8%) of 95 samples of SIL were associated with HPV. In 19 samples of squamous carcinoma and 9 of adenocarcinoma, the prevalence rate for HPV was, respectively, 84.2% (16 out of 19) and 55.6% (5 out of 9), type 18 being found in 100% of HPV-positive cases of adenocarcinoma.[34] Nagai et al. detected the prevalence of HPV in 89.9% of cases in squamous carcinoma (n=239), 93.8% of cases in adenosquamous carcinoma (n=17), and 51.4% of the samples in adenocarcinoma (n=37).[35] Lacey et al. analyzed 116 cases of adenocarcinoma and 129 samples of squamous carcinoma of the cervix, and found the presence of HPV in 82% of adenocarcinomas and in 73% of epidermoid carcinomas.[36] Andersson et al. found the presence of HPV in 76% of 82 cases of endocervical adenocarcinoma, with 45% of these associated with HPV18.[37]

The analysis of the results is consistent with a significant association between the presence of the HPV genome and immunohistochemical expression of Ki-67 (p=0.009), and no association with the expression of p53 and bcl-2. Positive immunohistochemical staining for p53, Ki-67 and bcl-2 was found in 122, 207, and 84 cases (53.27%, 90.39%, and 36.68%), respectively. An analysis of the molecular events involved in the carcinogenesis of the uterine cervix suggests that neoplastic cells possess an alteration or genetic mutation in the mechanism of apoptosis, so as to favor an increased survival in these cell groups. Protein p53 induces apoptosis through the activation of the bax gene and inhibition of bcl-2. Neoplasms expressing p53 by immunohistochemistry present some mutant form of this protein, which is unable to regulate the cell cycle. Despite some contradictory studies, a relationship has been reported between p53 expression and the development of SIL and cervical squamous carcinoma. It is probable that the

expression of p53 will increase during the progression of SIL to squamous carcinoma, and it can be considered as a good marker to evaluate neoplasms of the uterine cervix with squamous differentiation.[13-15,38-40] The expression of bcl-2 also appears to be a good histological marker of squamous cervical lesions associated with HPV. However, this study demonstrated that in endocervical adenocarcinoma the presence of HPV is related only to the expression of Ki-67, with no significant association with proteins p53 and bcl-2, or of Ki-67 with these two proteins. Thus, it seems that HPV can be an important cofactor for the development and progression of both squamous carcinoma and adenocarcinoma of the uterine cervix. In lesions with glandular differentiation, however, HPV seems to promote direct alterations in the mitosis and multiplication stages of the cell cycle, which can be estimated by the expression of Ki-67, inducing a higher cell proliferation index. In squamous carcinoma and SIL, HPV may be related to the presence of alterations in the mechanism of apoptosis, or to an increase in cell survival, which can be gauged through the immunohistochemical expression of p53 and bcl-2. Thus, the squamous or glandular differentiation of cells previously infected by HPV can predict the possible phenotypic and genotypic characteristics of lesions progressively induced by this agent.[10,18,19,22,41-43]

An inverse association between immunohistochemical expression of p53 and bcl-2 has been observed in cases of ductal carcinoma of the breast and ovary, lymphomas and adenocarcinomas of the colon and rectum. Protein p53 is not detected by immunohistochemistry in the normal endocervical mucosa, and its expression suggests an anomalous protein, with consequent alterations in the cell cycle and in apoptosis. The expression of bcl-2 has been associated, by some authors, with the alteration of p53 in cases of cervical squamous carcinoma associated with HPV types of high oncogenic risk. Cells infected with HPV16 and -18, and those presenting degradation of p53, are incapable of interrupting their cell cycle, and often present a genetic instability which alters the mitotic stage. The binding of E6 protein of HPV types of high oncogenic risk to p53 in cells infected with this virus results in a fast proteolytic degradation of p53, through a route dependent on ubiquitin, thus determining a loss in the repair mechanism of the cell genome during the normal cell cycle. The expression of viral E6 protein is even related to an anti-apoptotic effect, in part due to the degradation of p53. E6 enhances the activities of malignant transformation and immortalization of cells infected with HPV, which are promoted by E7 protein of the HPV types of high oncogenic risk. Physiologically, p53 interacts with promoter p34 and cyclin-B1, decreasing their expression, by binding with transcription factor NF-Y and regulating the G2/M stage of the cell cycle. In case of inactivation of p53, there is an accumulation of p34 and cyclin-B1, favoring the tumor cell to rapidly enter the stage of DNA synthesis and cell replication.[9,13,16-18,41,42,44,45] McCluggage et al. suggested that p53 mutation constitutes a late event in the progression of endocervical adenocarcinoma.[40] Park et al. observed positive immunohistochemical expression of p53 in 49 (58.3%) out of 84 cases of squamous carcinoma of the uterine cervix, and 35 cases were associated with the presence of HPV; however, they did not detect a significant relationship between protein expression and viral presence.[46] Toki et al. determined immunohistochemical expression of p53 in 3 out of 6 cases of endocervical adenocarcinoma of the minimum deviation subtype, with only one case associated with HPV type 16.[47]

Nair et al. analyzed 230 biopsies of the uterine cervix, and no case of normal tissue of the uterine cervix (n=40) showed positivity to p53, while p53 expression was positive in 9 cases of low-grade (n=37) and 43 cases of high-grade intraepithelial lesions (n=43), and in all of the cases of squamous carcinoma (n=110). The HPV genome was found in 5 samples of the uterine cervix with normal morphology at microscopy, 13 cases of low-grade and 29 cases of high-grade intraepithelial lesions, and 85 cases of squamous carcinoma. The authors reported a positive association between the immunohistochemical expression of p53 and the presence of viral DNA, an inverse relation between Ki-67 and bcl-2, and association of the presence of HPV with an increase in the rate of cell proliferation (increased expression of Ki-67) in all samples of the study.[31] Saegusa and Okayasu determined an immunohistochemical expression of Ki-67 of less than 5% in normal cervical epithelium (n=39), about 46% in adenocarcinoma *in situ* (n=18), about 30% in mucinous adenocarcinoma (n=31), and 50% in cases of endometrioid adenocarcinoma (n=31). By PCR technique they detected the presence of HPV in 46.9% of adenocarcinomas (30 samples), and an impressive association between the presence of HPV and Ki-67 expression.[48]

Previous works indicated a frequency of 15% to 40% of SIL associated with adenocarcinoma.[4,10,12,27,49,50] In the cases evaluated here, 72 squamous intraepithelial lesions (31.5%) were found to be occurring jointly with adenocarcinoma. The facts that 75% to 98% of the SIL are associated with HPV infection, and that the joint occurrence with adenocarcinoma is common,[4,10,12,24,27,49,50] support the hypothesis that certain types of HPV, probably type 18, are specifically correlated with the development of cervical neoplasms with glandular differentiation. A higher prevalence of HPV16 is reported in squamous cervical lesions, and a predominance of HPV18 in the glandular lesions of the uterine cervix. It is thus suggested that the initial infection in a particular cell group by a specific type of HPV can also be an important factor predicting glandular or squamous differentiation of neoplasms of the uterine cervix induced by HPV. It is supposed that HPV16 presents a tropism for intermediate filaments of the squamous cells of

the uterine cervix (cytokeratins of high molecular weight), which is favored by the expression of viral protein E4 and related to coilocytotic atypias of squamous intraepithelial cells, while HPV18 presents tropism for cells with glandular differentiation, or for cells of the squamous-columnar junction, which possess, in their makeup, cytokeratins of lower molecular weight and the presence of intracytoplasmic mucin.[4,10,12,24,27,49,50]

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