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Original Article

p16^{INK4a} and Ki67 expression in normal, dysplastic and neoplastic uterine cervical epithelium and human papillomavirus (HPV) infection☆

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ABSTRACT

Cellular cycle proteins like the p16^{INK4a} and the Ki67 proliferation nuclear antigen have been used as oncogenicity cellular markers. The E6 and E7 oncoproteins interact with tumor suppressor genes p53 and pRb, culminating with the p16^{INK4a} overexpression.

The objective of this study was to evaluate the presence of HPV-DNA in 174 cervical biopsies and correlate the different histological grades with the p16^{INK4a} and Ki67 immunohistochemical expression (IHC).

A cross-sectional study that enrolled a total of 174 women who underwent uterine cervical biopsies between February 2003 and December 2006, in southern Brazil, was performed. Cervical smear samples were analyzed for the presence of HPV-DNA through polymerase chain reaction (PCR), and biopsy samples were examined for p16^{INK4A} and Ki67 expression through IHC techniques.

The presence of HPV-DNA was observed in 89% of the tested patients, among which 52% were positive for high-risk (HR) viral types [16, 18 and 31]. Regarding p16^{INK4a}, an expression of 69% was observed, being expressed in 100% of the high-grade squamous lesions (HSIL) and HR-HPV-DNA positives. Ki67 expression was associated with the lesion grade, being more expressive in the most severe lesions ($p < 0.001$). p16^{INK4A} and Ki67 markers coexpression was present in 86% of the samples ($p < 0.001$), being 100% among those positive to HR-HPV-DNA with HSIL ($p < 0.001$).

The results suggest an association between the presence of HR-HPV infection and the p16^{INK4a} and Ki67 expression and which is even stronger among women with HSIL.

Introduction

During the last 50 years, the incidence of cervical carcinoma has declined because of the screening programs. Nevertheless, it is still the third most common malignancy after breast and colorectal cancer, and is a relevant cause of mortality in women around the world [47]. Persistent infection with high-risk human papillomavirus (HR-HPV) represents the main risk factor associated with cervical carcinogenesis, which was observed in up to 99% of reported cases. However, HPV infection is a central and necessary, although not sufficient, cause of cervical cancer [3,33].

Epidemiological studies on genital human papillomaviruses infection in general population are crucial for the implementation of health policy guidelines for developing the strategies to prevent primary and secondary cervical cancer [3,17]. More than 40% of women are infected by HR-HPV sometime in their life, where 25% of them develop persistent infections and one third can evolve to high-grade intraepithelial lesion within 5–6 years after initial infection [20].

The cytomorphological criteria for the diagnosis of preneoplastic lesions present a wide inter- and intraobserver variability, thus justifying the need to link immunohistochemical markers (IHC) for the diagnosis optimization [25,46].

Besides viral infection, other factors such as host susceptibility and ease of spread of cancer cells are factors that facilitate the malignant transformation process [9,20,39].

In normal cells, the cyclin-dependent kinases (CDKs) activity is regulated by the CDK inhibitors, including the p16^{INK4a}, which acts

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as a tumor suppressor inhibiting the CDK4 and CDK6 that phosphorylize the retinoblastoma protein (pRb). The $p16^{INK4a}$ function loss is analogous to the pRb deactivation in cells with virus-induced immortality, such as HPV. p16 regulates the cell cycle and cell proliferation by inhibiting cell cycle G1 progression [12,18,26].

The $p16^{INK4a}$ protein induction is a present factor in the premalignant and malignant lesions caused by the uterine cervix HPV and have been considered as a dysplasia marker [29,45]. Several authors have described the $p16^{INK4a}$ role in the uterine cervical neoplasias to confirm equivocal cytological results, for its importance linked to the HPV-test, as a diagnostic tool, or in the prognostic analysis of lesions [11,16,45]. The $p16^{INK4a}$ activity has been studied in other tumor types or even linked to other markers in order to elucidate diagnoses [28,30,50].

The Ki67 antibody expression has been related to various types of malignant lesions, such as vulva, penis, breast, and uterine cervix, indicating the presence of mitotic activity [21,22,33,55,57]. It is a non-histone cell proliferation antigen, present in the cell nucleus, which is expressed in all the cellular cycle phases, except in G0 [57]. The Ki67 expression in t he lower third of the basal layer of the metaplastic epithelium is an event that occurs in active lesions and is a strong predictor of proliferation and progression, increasing its expression in parallel with the lesion aggressiveness [33].

In the present, study uterine cervix biopsies of patients attending two Public Hospitals were analyzed with the purpose to verify the expression of $p16^{INK4a}$ and Ki67 markers in different cervical lesion grades and also in the presence of HPV infection.

Materials and methods

A cross-sectional study enrolling 174 women submitted to uterine cervical biopsies between February 2003 and December 2006 was performed. All participants attended one of two Public General Hospitals in southern Brazil, where they were referred after presenting an abnormal cervical cancer screening results. All of them presented the histopathological diagnosis performed at one of the hospitals. The study was approved by the Ethical Committee of the Hospitals, and all information was collected after informed consent from all participants.

All biopsied women were examined through IHC techniques. In one of the hospitals, HPV-DNA through polymerase chain reaction (PCR) test was in the routine examination, so 83% (145) women of the present study were tested. The positive HPV-DNA samples were typed for the most prevalent HR-HPV in Brazil (HPV-16, 18 and 31).

The extraction of DNA was carried out according to the Proteinase K protocol [49]. The technique was directed to the L1 gene and the primers used were My09 and My11. The PCR conditions were described by Bauer et al. (1993) and Coutlée et al. (2002) [5,13]. For the PCR reaction internal control, ß-globin primers were developed and tested in all samples in order to verify the viability of tested DNA [6].

All the positive samples in the HPV-DNA were typed for HR-HPV (HPV-16, -18 and -31), using specific primers corresponding to the E6 and E7 regions of the viral genome. The amplification conditions and the primers used are based on the methodology described by Cuzick et al. (1994) [14]. In all the tested samples, a multiplex PCR was performed using My09/My11 primers corresponding to L1 gene of HPV viral genome primers complementing human β-globin gene (gH20 e PC04). β-globin primers were used in these reactions in order to verify the viability of tested DNA.

After the selection of the biopsied paraffin blocks, new slides were prepared and stained through the hematoxylin-eosin technique for the histological revision [24]. All slides were reviewed independently by two trained pathologists (MIE; LM) and blind to the previous diagnosis. Any disagreement (<1%) was solved by consensus.

The 174 cervical biopsies fixed in formalin, embedded in paraffin were cut out (4 μm) and deparaffinized in xylene, followed by rehydration. For the antigen retrieval, the slides were heated in citrate buffer pH 6.0 in a microwave for 20 min. The sections were placed at room temperature and washed with phosphate buffer three times for 5 min each. After the endogenous peroxidase block, the sections were rehydrated and the slides were submitted to the immunohistochemical process [37,51].

In order to determine the $p16^{INK4A}$ expression, the $p16^{INK4A}$ antibody was used (Neomarkers Ab-7, 16P07 clone, cat#AP-9003, CA, USA) through the technique described above [1]. The cytoplasmatic and nuclear staining were evaluated and considered as positive for the $p16^{INK4a}$ expression. The expression intensity was graduated according to Bulten et al. (2006) and was considered as negative (−) if none of the cells expressed staining, positive (+) if the percentage of expressing cells ranged between 1 and 25%, positive (++) if the percentage ranged between 26 and 75% and positive (+++) if over 75% of the cells were stained [7]. The standard expression was also evaluated and considered as focal if the expression was concentrated in some areas, and diffuse, if the expression was distributed throughout the slide.

The antibody Ki67, MIB-1 clone (DAKO, Glostrup, Denmark), was incubated for 12 h, at 4 °C, at the dilution of 1:200, followed by the application of the streptavidine-biotine-peroxidase complex (LSAB, Dako), revealed with diaminobenzidine tetrahydrochloride (DAB Kit, Dako) and contrasted with hematoxylin. The reaction presented as a positive control for Ki67, a cutting containing reactive hyperplasic lymphonode, where the negative control was performed in the same type of sample without the primary antibody. The expression of Ki67 was considered positive when more than 5% of the basal cells present nuclear staining.

The study's outcomes were the histopathological diagnosis of the uterine cervix lesions, the HPV-DNA and HR-HPV-DNA typing HPV (-16, -18, -31). The results of the histopathological examination were categorized into normal, low-grade squamous lesion (LSIL) and high-grade squamous lesion (HSIL). The LSIL category included the results with grade 1 cervical intraepithelial neoplasia (CIN I) diagnosis; and the HSIL category, included the findings with grade 2 and 3 cervical intraepithelial neoplasia (CIN II, III) and the other is invasive cancer diagnosis [59]. The HPV-DNA results were classified into two categories: presence or absence of HPV and/or HR-HPV. The $p16^{INK4a}$ and Ki67 expressions were related to such outcomes.

In order to compare the categorical variables, the Chi-square test or Fisher's Exact test were used, when recommended. The continuous variables were analyzed through the Student's t test and ANOVA. The findings with $p < 0.05$ were considered as statistically significant. For data analysis, the SPSS Program Version 16.0 was used.

Results

The women studied were, on average, 38.5 ± 13.2 years old. When considering the lesion histological grade, the mean age of women with LSIL and HSIL was 34 ± 12.5 and 39 ± 12 old, respectively ($p = 0.03$).

Among the patients tested for HPV-DNA, 83% were positive. From those, 79% were also tested for HR-HPV-DNA, and 55% resulted positive. Among HR-HPV-DNA, 33%, 15% and 8% were the frequencies observed for HPV16, HPV31 and HPV18, respectively. Table 1 describes the distribution of HPV-DNA according to the anatomopathological diagnosis.