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Expression of cyclooxygenase-2 (COX-2) and Ki67 as related to disease severity and HPV detection in squamous lesions of the cervix

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Abstract

Objectives. To assess the expression of cyclooxygenase (COX-2) and Ki67 in cervical squamous lesions in relation to disease severity and human papillomavirus (HPV) detection.

Subjects and methods. For this cross-sectional study, 223 women subjected to diathermic conization of the cervix have been enrolled, between February 2001 and April 2004. All patients undertook pelvic examination, including colposcopy and collection of samples for Hybrid Capture II (HCII). Pathological assessment disclosed: 9 cases of normal epithelium/cervicitis, 33 CIN1, 28 CIN2, 146 CIN3 and 7 invasive squamous cell carcinomas. COX-2 and Ki67 protein expression was determined with immunohistochemistry. COX-2 immunoreactivity grading was based on the German ImmonoReactive score. The continuum percentage of positive cells was used for the assessment of nuclear Ki67 expression.

Results. Expression of COX-2 did not correlate with disease severity and with Ki67 expression. The HPV detection rates did not differ significantly across COX-2 protein expression strata, ranging from negative to strong expression. Ki67 expression, however, was higher in the CIN3 group ($P = 0.001$) as compared to the specimens rendered as normal/cervicitis.

Conclusions. COX-2 protein expression did not correlate with disease severity or Ki67 expression.

Keywords: MIB-1; Cervical intraepithelial neoplasia; Cancer; Cervix; Immunohistochemistry

Introduction

Cancer of the uterine cervix is still one of the prime concerns in public health, mainly in the developing world. The disease is preventable and has its etiology firmly correlated with human papillomavirus (HPV) infection [1]. The treatment of its pre-invasive form, the cervical intraepithelial neoplasia (CIN), can be easily accomplished. In recent years, several biologic markers or indexes have been studied as potential tools to determine the prognosis and biological behavior of various types of neoplasia, and immunohistochemistry (IHC) is probably the most affordable and simple technology to detect many such biomarkers. This fact prompted investigators to develop and test antibodies in a widespread range of neoplasic lesions, and gynecological pathology has not escaped this tendency [2,3].

Oncogenes and cell-cycle regulators suggested to play a role in genesis of cervical cancer include c-erb-2/neu, p27, p53 and p16INK4a and Ki67 [4]. In very simple terms, the expression of these markers is affected by the disrupting effects of HPV E6 and E7 oncogenes on the cell-cycle control mechanisms, leading to uncontrollable cell proliferation. Ultimately, cell multiplication can be assessed by detecting the antigen Ki67, expressed in the nuclei of proliferating cells. The rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins in inflammatory processes, cyclooxygenase-2 (COX-2), has also been shown to correlate with carcinogenesis in humans [5]. Firstly studied in esophageal squamous tumors, COX-2 has also been demonstrated to be over-expressed in breast, stomach, colon and cervical neoplasms as well. It is suggested that COX-2, besides its participation in inflammatory responses, might

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induce tumor proliferation and spread by enhancing mitogenesis, reducing cellular adhesion and immune surveillance [6]. In cervical cancer, comparative studies found that COX-2 might be prognostic, relating to a diminished response to radiotherapy, in terms of survival, and to an augmented resistance to chemotherapy [7,8]. However, the behavior of COX-2 in pre-invasive cervical lesions is still under investigation, and its expression has been suggested to increase in parallel to the grade of CIN and possibly to have a potential in help predicting CIN recurrence after conservative treatments (e.g. diathermic or cold-knife conization) [9]. Nevertheless, the number of studies available in literature remains limited, and discrepant results have been reported.

In order to shed further light on the understanding of COX-2 in the pre-invasive forms of squamous cervical neoplasia, we decided to conduct this study, aimed at assessing the expression of COX-2 in different grades of squamous lesions of the uterine cervix and its possible relations to nuclear expression of Ki67 and HPV detection.

Materials and methods

Selection of the women and examination routine

For this cross-sectional study, 223 non-consecutive women subjected to diathermic conization of the cervix were enrolled. Women had been referred to the colposcopy clinics of Campinas State University, Brazil, due to abnormal findings in their cervical cytology smears (Pap tests). All patients were subjected to an interview, addressing clinical and socio-demographic concerns, and shortly afterwards to a thorough pelvic examination, including colposcopy and collection of samples for HCII. The decision to perform diathermic conization as the primary treatment was made on the basis of the cytology result coupled with the clinical/colposcopic aspect of the cervix. Enrollment began in February 2001 and ended April 2004. The study has been approved by the local ethics committee, and all patients signed the Informed Consent Form.

HPV detection

The specimens for HCII were tested with probe B (high-risk HPVs: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) [10], and the tests were classified at the relative light unit/positive control (RLU/PC) ratio (RLU of specimen/mean RLU of two positive controls) of 1 pg/ml or greater. These RLU/PC ratios provided an estimate of the amount of HPV-DNA in the specimens, i.e. the viral load in the sample. The storage of the specimens and all reagents as well as conduction of the tests took place at the Campinas State University Medical School Hospital Laboratory, following the manufacturer's instructions (Digene Diagnostics Inc., USA).

Histology

Histological samples consisted of 223 diathermic conization specimens, which were fixed in 10% phosphate-buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E). Samples for histology were analyzed according to the World Health Organization criteria [11] and classified as normal/cervicitis (9 cases), CIN1 (33 cases), CIN2 (28 cases), CIN3 (146 cases) and invasive squamous cell carcinoma (7 cases).

Immunohistochemistry (IHC)

IHC reactions were performed on slides obtained from the tissue blocks that harbored the most significant lesion, as detected with H&E assessment. Five-micrometer sections were obtained from the tissue blocks and transferred to silanized glass slides and dried overnight at 37°C. The sections were deparaffinized using a sequence of xylene followed by washes in graded ethanol and phosphate-buffered saline (PBS; pH 7.4). Pretreatment of the slides consisted of vapor "steam cooking" for 30 min in 10 mmol/L sodium citrate (pH 7.0). After washing the slides with PBS, endogenous peroxidase was blocked by 3% hydrogen peroxide (H_2O_2), by three sequential incubation baths, 3 min each. Samples were then incubated for 30 min in a humid chamber at 37°C with the antibodies: anti-COX2 (1:100 dilution) or Ki67 (1:150 dilution). Afterwards, slides were incubated overnight at 4°C. Slides were then incubated with Envision® (DAKO A/S, Copenhagen, Denmark) for 30 min and with a solution of avidin biotinylated peroxidase. For slides development, a diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate was used for 6 min at 37°C. The slides were washed with water and counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted with resin Entellan®. Slides with colitis known to be positive for the COX-2 were used as positive controls. The corresponding sections without the primary antibody were employed as negative controls.

Assessment of IHC slides

Reading of all slides was performed by one investigator, who was blinded to the H&E diagnosis rendered by a pathologist and to the HPV status of the patients. Digitalized photographs of high-power fields (400×) were taken from hot-spots of reaction with a Nikon COOLPIX Camera 995, and then images were ported to a personal-computer-based software for histological analyses (Imagelab 2000).

COX-2 protein expression

COX-2 immunoreactivity grading was based on the German ImmunoReactive score [12]. Firstly, staining intensity in the cytoplasm was rated on a scale from 0 to 3, with 0 being no staining at all, 1 weak staining, 2 moderate and 3 strong staining. Then, positive and negative cells were counted, with no less than 500 cells as the minimum acceptable count number. The percentage of positive cells was then scored as: no staining as 0; 1–10% as 1; 11–50% as 2; 51–80% as 3; and 81–100% as 4. The final score was calculated by multiplying the score obtained with the staining intensity by that derived from the percentage of positive cells, achieving theoretical results ranging from 0 to 12. A final score of 0 was regarded as negative, 1–4 as weak, 5–8 as moderate and 9 to 12 was considered as strong immunoreactivity. COX-2 protein expression was negative in 35 (15.7%) histological specimens, whereas 70 (31.4%) lesions presented with weak expression, 50 (22.4%) with moderate and 68 (30.5%) with strong expression of the antigen. For statistical purposes, two groups were formed: negative and weak immunoreactivity (67 women); moderate and strong immunoreactivity (98 women).

Ki67 nuclear expression

A continuum percentage of cells expressing Ki67 in their nuclei were used for the assessment of cell proliferation rate. A minimum count of 500 nuclei was mandatory. Nuclear staining of basal cells was considered as the internal positive control of reaction. Specimens of lung adenocarcinoma, known to be strongly positive for Ki67, were used as external reaction positive controls (Fig. 1).

Statistical analysis

A logistic regression model, adjusting for the pathological categories, has been applied to evaluate a possible relation between HPV detection and COX-2 expression. Odds ratios were calculated by exponentiation of the correlation coefficients. A generalized linear model, also adjusted for the pathological assessment, has been used to determine possible correlations between Ki67 expression—reflecting the cell proliferation rate—and COX-2 protein expression. Another logistic regression model has been fit—adjusted for the HPV status of the women—to investigate a possible relation between histological grade of the disease and COX-2 protein expression. The evaluation of Ki67 expression as related to the pathological categories has been carried out through a generalized linear model, with adjustment for the HPV detection. Data were recorded in an OpenOffice® spreadsheet, and statistical treatment has been