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Original Article

Cox-2, EGFR, and ERBB-2 Expression in Cervical Intraepithelial Neoplasia and Cervical Cancer Using an Automated Imaging System

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Summary: We hypothesized that the activation of cyclooxygenase (COX)-2, epidermal growth factor receptor (EGFR), and ErbB-2 signaling is required for cervical intraepithelial neoplasia (CIN) lesions to progress to cervical cancer. A retrospective analysis was performed in 179 patients with Stage I squamous cell carcinoma (SCC) and 233 patients with CIN (112 CIN I, 47 CIN II, and 74 CIN III). COX-2, EGFR, and ErbB-2 expression was analyzed by immunohistochemistry using the ACIS III automated imaging system. The mean expression of COX-2, EGFR, and ErbB-2 was compared between the various stages of CIN and SCC. COX-2 mean expression was predominantly cytoplasmic, increasing significantly from CIN I to CIN II, CIN III, and SCC ($P < 0.001$). EGFR mean expression also rose significantly during tumor progression from CIN I to SCC ($P = 0.001$). CIN I samples were negative for ErbB-2 expression. CIN II, CIN III, and SCC were considered positive for ErbB-2 expression in 2.2%, 14%, and 16.2% of cases, respectively. There was also a statistically significant correlation between increase of ErbB-2 positivity from CIN to SCC. We conclude that COX-2, EGFR, and ErbB-2 expression increase significantly during the progression of CIN to cancer. **Key Words:** Cervical intraepithelial neoplasia—Uterine cervical neoplasm—EGFR—Cyclooxygenase 2—ERBB-2.

Invasive cervical cancer constitutes 15% of cancers in females and ranks first or second among cancers (1,2). The causal relationship between human papillomavirus (HPV) and invasive cervical cancer is supported by epidemiological and molecular data (3,4).

The development of cervical cancer from cervical intraepithelial neoplasia (CIN) can be prevented when the precursor forms are diagnosed and treated early. Therefore, the recognition of molecular changes that result from dysregulated activity of the E6 and E7 proteins during HPV infection might aid in the identification of lesions that are more likely to progress and lead to novel disease prevention methods and therapeutics (5–7).

Cyclooxygenase (COX)-2 converts arachidonic acid in the cytoplasmic membrane into prostaglandin

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The authors declare no conflict of interest.

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H2 and, subsequently, to prostaglandin E2 (PGE2). PGE2 regulates cell proliferation, differentiation, and apoptosis through several autocrine and paracrine signaling pathways (8).

Epidermal growth factor receptor (EGFR/ErbB1) and ErbB2/HER2 are subtype I tyrosine kinases. Protein kinases regulate nearly every aspect of cell biology (9,10).

In *in vitro* models of HPV16-mediated carcinogenesis, the increases in EGFR expression at various stages of HPV-induced transformation regulate immortalization and conversion to the malignant phenotype (11–13).

Yasmeen et al. (14) reported that E6/E7 of HPV type 16 cooperates with ErbB-2 to induce transformation in human oral epithelial cells. However, the function of ErbB-2 receptor in cervical cancer is unknown. Narisawa-Saito et al. (15) evaluated the effects of the E6 and E7 genes of HPV type 16 on ErbB-2 expression in immortalized human cervical keratinocytes and demonstrated the involvement of HPV type in the oncogenic regulation of ErbB-2.

In *in vitro* models of carcinogenesis, HPV type 16 E6 and E7 oncoproteins stimulate COX-2 transcription by activating the EGFR-Ras-MAPK-AP-1 pathway. Further, COX-2 has been reported to be a direct target of ErbB-2 (16). Recent evidence of crosstalk between EGFR and COX-2 has also been described (17). Thus, the complex interplay between signaling pathways, entailing extensive feedback regulation and multiple levels of crosstalk, facilitates carcinogenesis.

We hypothesized that in a multistep model of the carcinogenesis of cervical cancer, the activation of COX-2, EGFR, and ErbB-2 signaling is necessary for CIN lesions to progress to cervical cancer.

We analyzed COX-2, EGFR, and ErbB-2 expression in uterine cervical cancer by immunohistochemistry and precancerous lesions using an automated cellular imaging system (ACIS III) to obtain a more objective and reproducible interpretation of the immunohistochemistry results.

MATERIALS AND METHODS

Patients Characteristics

Our retrospective analysis included 472 individuals admitted to the Departments of Gynecologic Oncology, A.C. Camargo Cancer Hospital and Brazilian Institute of Cancer Control, from January 1985 to December 2001. Paraffin blocks were retrieved from the archives, and the pathology slides were reviewed. They were classified per World Health Organization criteria as CIN I, CIN II, CIN III, and squamous cell carcinoma (SCC). A total of 412 cases had paraffin-embedded tissues that were suitable for immunohistochemical analysis. The remaining 60 patients were excluded.

The final sample comprised 179 patients with FIGO (International Federation of Gynecology and Obstetrics) Stage I SCC and 233 cases of CIN (112 CIN I, 47 CIN II, and 74 CIN III). All patients with Stage I SCC underwent radical hysterectomy; no patient received neoadjuvant treatment. Clinical information was obtained from medical records. The Institutional Review Boards of both institutions approved the study.

Tissue Microarray Construction

The 233 cases of CIN were examined by histology, and a tissue microarray was constructed from selected areas of 179 SCC samples. Two tissue cores (1 mm in diameter) were sampled from each marked tumor area on the donor block and mounted into a recipient paraffin block on a custom-made instrument (Beecher Instruments, Silver Springs). Cores were spaced at intervals of 0.2 mm.

Immunohistochemical Staining

Three-micrometer sections of the tissue microarray were transferred to an adhesive-coated slide (Instrumentics Inc., Hackensack). The slides were deparaffinized, rehydrated, and then subjected to antigen retrieval (citrate pH 6.0). The primary anti-COX-2 (NCL-COX-2) (titer, 1:6000) was purchased from Novocastra Laboratories (LTDA, New Castle, UK). The primary anti-EGFR (M3563) (titer, 1:400) and anti-ErbB2 (anti-human c-erbB-2 oncoprotein—HercepTest) (titer, 1:3000) were purchased from Dako Corporation (Carpinteria, CA).

Briefly, the sections were incubated in 3% aqueous hydrogen peroxide for 20 minutes to quench endogenous peroxidase activity and with phosphate-buffered saline 10 mM pH 7.4 for 5 minutes at room temperature to suppress nonspecific binding of subsequent reagents. The reaction was followed with incubation of the primary antibodies in phosphate-buffered saline with bovine albumin 1% (Sigma, A9647, EUA) and NaN_3 0.1%, for 18 hours at 4°C. The antigen-antibody complexes were incubated with postprimary block, NovoLink Polymer (NovoLink Max Polymer, #RE7260-k, UK) for 30 minutes at 37°C and followed by incubation with: 3,3′ diaminobenzidine tetrahydrochloride 60 mg%

(Sigma, D-5637, EUA); of dimethylsulfoxide (DMSO) 1 mL; H_2O_2 6% 1 mL; phosphate-buffered saline 100 mL, for 5 minutes at 37°C, in dark place. The sections were then counterstained with Harris hematoxylin, dehydrated and mounted with a glass coverslip and xylene-based mounting media (18).

COX-2, EGFR, and ErbB-2 for patients with cervical cancer were examined by immunohistochemistry using duplicate slides at 2 depths of the tissue microarray—separated by 25 sections (at least 125 μm)—representing 2-fold redundancy for each case.

Whole-section CIN slides was subjected to immunohistochemistry using the same method.

Negative controls were performed by incubation of the tissue sections with nonimmune serum. Positive controls were used according to the manufacturer's recommendations (19,20).

Immunohistochemical Analysis

The slides were placed in the ACIS automated imaging system (ACIS III DAKO), and the stains were quantified as described (19–21). Briefly, a robotic microscope scanned each slide, and the ACIS III captured images from each slide, quantified the staining intensity within a selected region, and calculated a numerical score. The system quantified membranous ErbB-2 and EGFR expression and cytoplasmic COX-2 staining.

To analyze immunohistochemical EGFR expression, the *membrane histo* program was used to measure optical membrane density, and the *cytoplasm histo* program was used to evaluate cytoplasmic COX-2 expression. The *herceptest* program was used to assess immunohistochemical ErbB-2 expression.

The operator quantified at least 5 areas with the highest staining intensity, as recommended (ACIS III DAKO). The selected areas were restricted to the epithelium for cervical cancer and to the dysplastic cells for CIN. The system recognizes 256 levels of intensity and calculates fractional scores for selected areas, generating an average score for all areas (19,20). A mean value was obtained from the 2 cores or 2 whole-section slides for each patient with cervical cancer or CIN, respectively.

The manufacturer recommends that cases with an average score of 2.2 or higher are considered to positive for ErbB-2 expression, whereas cases with average scores that are lower than 2.2 do not express the protein. This cutoff has the higher relationship between immunohistochemical expression and presence of gene amplification (22).

Statistical Analysis

The associations between mean immunohistochemical intensity scores for COX-2 and EGFR between CIN I, CIN II, CIN III, and SCC were analyzed by nonparametric Kruskal-Wallis test. The ACIS software provides a parametric score that corresponds to the immunohistochemical HercepTest, and the association of immunohistochemical ErbB-2 expression (negative and positive) between CIN I, CIN II, CIN III, and SCC were analyzed by χ^2 test. For all tests, an α error up to 5% ($P<0.05$) was considered statistically significant.

RESULTS

The mean immunohistochemical expression score for COX-2 in CIN I, CIN II, CIN III, and SCC was 88.1 (SD = 8.63), 108 (SD = 11), 132 (SD = 17.3), and 161.6 (SD = 13.3), respectively, correlating significantly with CIN grade/SCC ($P<0.001$) (Fig. 1). COX-2 expression was predominantly cytoplasmic, increasing throughout the transition of CIN I to CIN II, CIN III, and SCC (Fig. 2).

Mean immunohistochemical EGFR expression in CIN I, CIN II, CIN III, and SCC was 102.1 (SD = 10.9), 111.5 (SD = 8.41), 118.4 (SD = 12.7), and 123.2 (SD = 20.3), respectively (Fig. 3). EGFR mean staining intensity rose significantly during the progression from CIN I to SCC ($P = 0.001$) (Fig. 4). This progression was associated with the gradual expansion of EGFR-expressing cells away from the basal layer and with increased intensity per cell.

ErbB-2 stained the cytoplasmic membrane, outlining the entire circumference of cells (Fig. 5). ErbB-2 was not expressed in CIN I, but 2.2%, 14%, and

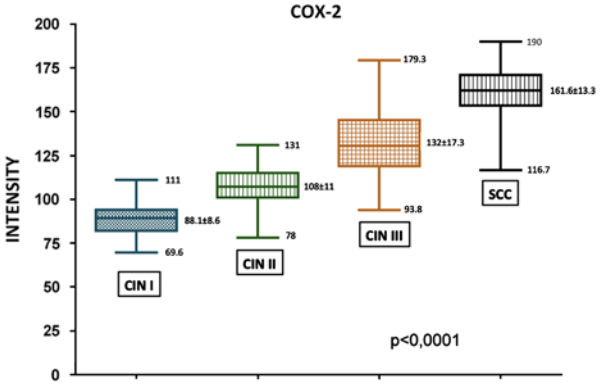

FIG. 1. Box plots of mean, SDs, minimum and maximum values of COX-2 immunohistochemical expression in CIN I, CIN II, CIN III, and SCC. Kruskal-Wallis test. $H = 374.8$; $P<0.0001$. CIN indicates cervical intraepithelial neoplasia; COX-2, cyclooxygenase-2; SCC, squamous cervical carcinoma.

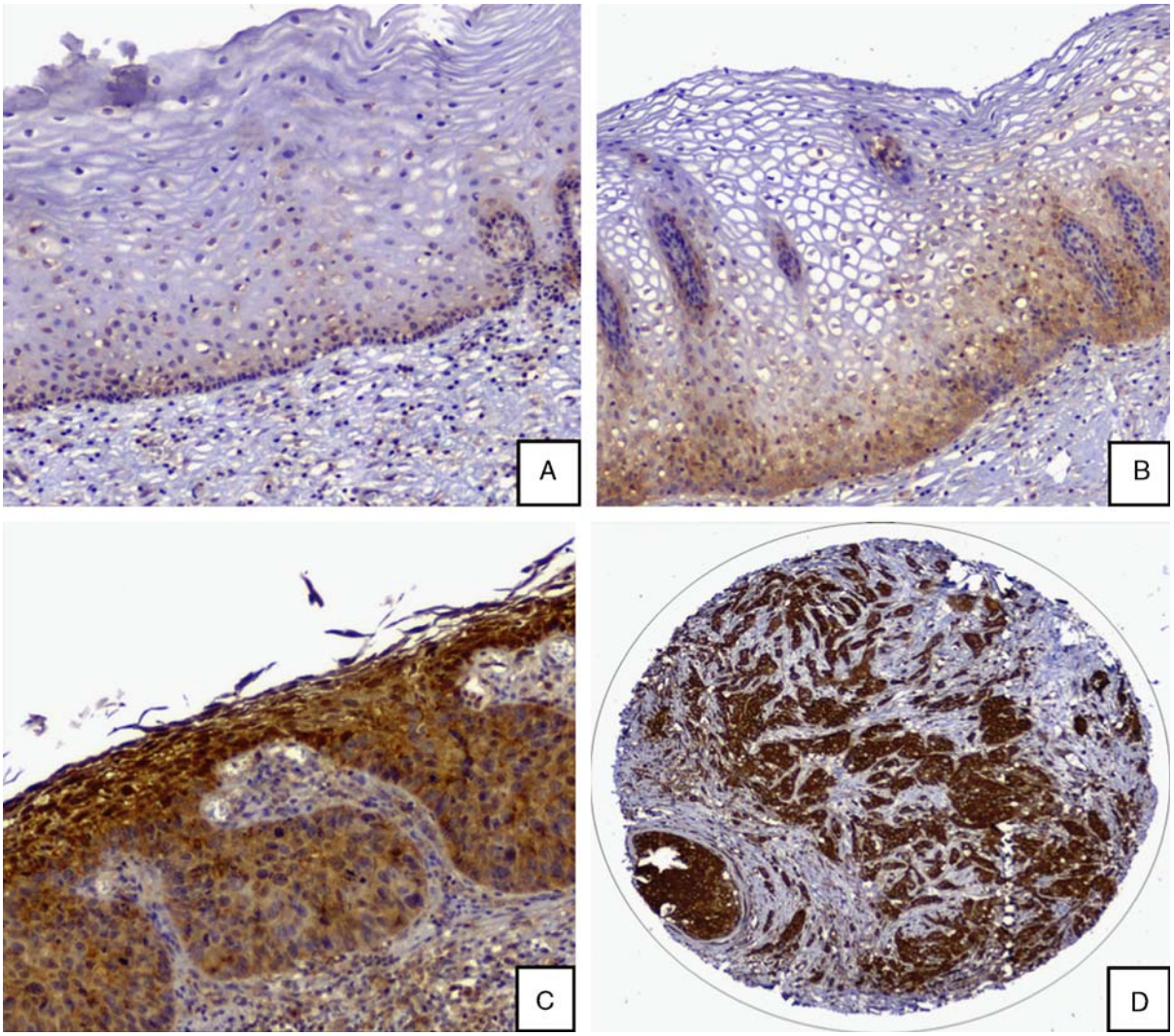

FIG. 2. (A) Normal cervical epithelium. (B) Microphotograph aspect of COX-2 cytoplasmic immunohistochemical expression in CIN I (10 ×). (C) COX-2 expression in CIN III (10 ×). (D) Low-power microphotograph aspect of COX-2 expression in SCC (4 ×). CIN indicates cervical intraepithelial neoplasia; COX-2, cyclooxygenase-2; SCC, squamous cervical carcinoma.

16.2% of CIN II, CIN III, and SCC cases, respectively, were positive. Despite the low frequency of ErbB-2 expression, there was a statistically significant correlation between intensity and the transition from CIN to SCC ($P<0.001$).

DISCUSSION

The causal role of persistent HPV infection in CIN and cervical cancer development is well established (23–25). However, not only high-risk HPV presence is sufficient to justify CIN progression, but also 16% of high-risk HPV-infected CIN1 or CIN2 women develop higher grade CIN (26). Thus, other factors must contribute to CIN progression, and either progression or prediction markers have been subject of intense study.

The potentially promising markers cover a wide variety of molecules in different classes, including cell adhesion, invasion, angiogenesis, metastasis, cellular receptors, cell proliferation, transcription, cell cycle regulation, apoptosis, and signaling pathways (27–33).

Keating et al. (34) have shown that cell cycle-related biomarkers, such as Cyclin A, E, p16, and others correlate with the degree of CIN lesions. Also, Kruse et al. (35) suggested in a series of 90 CIN that combined expression of Ki67, Rb, CK13, and CK14 gives accurate information of CIN progression risk.

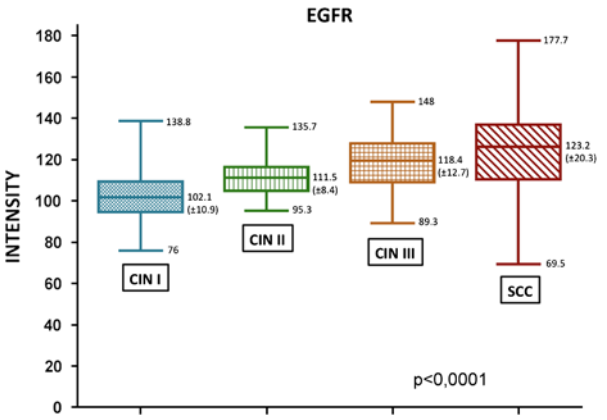

FIG. 3. Box plots of mean, SDs, minimum and maximum values of EGFR immunohistochemical expression in CIN I, CIN II, CIN III, and SCC. Kruskal-Wallis test. $H = 118.2$; $P < 0.0001$. CIN indicates cervical intraepithelial neoplasia; EGFR, epidermal growth factor receptor; SCC, squamous cervical carcinoma.

In an interesting study, Branca et al. (36) analyzed 13 markers to assess whether an individual marker or their combined expression would be an independent predictor of high-grade CIN and high-risk HPV infection. They found that the important predictors of CIN2 (and above) lesions were VEGF-C, laminin receptor-67, and PCNA. In addition, the most important predictors of high-risk HPV type were p16INK4a, survivin, and human telomerase reverse transcriptase.

Type I tyrosine kinase receptors and the signal transduction pathways in which they participate have critical functions in cancer biology, but the data regarding the immunohistochemical expression of EGFR and ErbB2 in cervical cancer are conflicting. The expression of COX-2 in cervical cancer has also been examined, yielding contradictory results.

Subbaramaiah and Dannenberg (37) suggested that COX-2 transcription is regulated by the E6 and E7 proteins of HPV16 through EGFR signaling. Because prostaglandin decreases the immunologic response against viral antigens, COX-2 and PGE2 overexpression might contribute to the persistence of high-risk HPV (38). Other interactions between COX-2 and EGFR have also been reported (39,40).

Farley et al. (41) observed immunohistochemical COX-2 expression in 32% of normal tissue samples, 50% of CIN I, 42% of CIN II, and 68% of CIN III. Sarian et al. (42) reported moderate or strong immunohistochemical COX-2 expression in 39.4% of CIN I, 50% of CIN II, and 57.5% of CIN III cases but concluded that there was no significant difference in expression across histologic strata. Dursun et al. (43) and Kim et al. (44) noted a lower incidence of COX-2 expression—24% and 26.7% of cases, respectively—in CIN III. In SCC, 28% to 57% of cases express COX-2 by immunohistochemistry (43–46).

In contrast to other studies, we did not categorize immunohistochemical COX-2 expression as negative or positive. Instead, we compared mean COX-2 expression between various stages of CIN progression and SCC in a relatively large series.

According to our findings, COX-2 expression is significantly associated with the higher grades of CIN and SCC, and we may propose that COX-2 is a potential marker for the late phases of CIN and cancer.

EGFR is expressed in epidermal keratinocytes and has central functions in repair, proliferation, and differentiation. EGFR dysregulation, however, can induce squamous metaplasia, epithelial hyperplasia, and progression to cancer (47).

Maruo et al. (48) reported EGFR expression in 75% of CIN I, 100% of CIN II, and 80% of CIN III cases. Mathur et al. (49) found 80% EGFR expression for all stages of CIN. In SCC, EGFR is expressed 25.8% to 72% of cases (45,48,50,51).

Similar to COX-2, we compared the mean EGFR expression between various stages of CIN and SCC. We noted that EGFR staining intensity rose significantly from CIN I to SCC. Nevertheless, the increase in EGFR expression varied between individual cells and the expanding cells that overexpressed it in whole tissue (52).

Berchuck et al. (53) reported equal EGFR expression between cancerous and normal basal epithelial cells, increasing proportionally in epithelium in CIN. They proposed that EGFR expression in squamous epithelium is a hallmark of proliferating keratinocytes.

We observed that EGFR expression increased due to progressive expansion of EGFR-expressing cells away from the basal layer and increases in EGFR expression per cell. We attribute this finding to the accuracy of the ACIS in detecting minimal differences in EGFR expression.

In the last phase of carcinogenesis, cancer cells acquire the capacity to invade adjacent structures (54–56). Epithelial-mesenchymal transition in cervical cancer correlates with overexpression of EGFR (57), and the interaction between the EGFR and COX-2 pathways might influence this process (58–60). Akerman et al. (12) also observed that COX-2 expression is associated with the activation of EGFR signaling in cervical cancer.

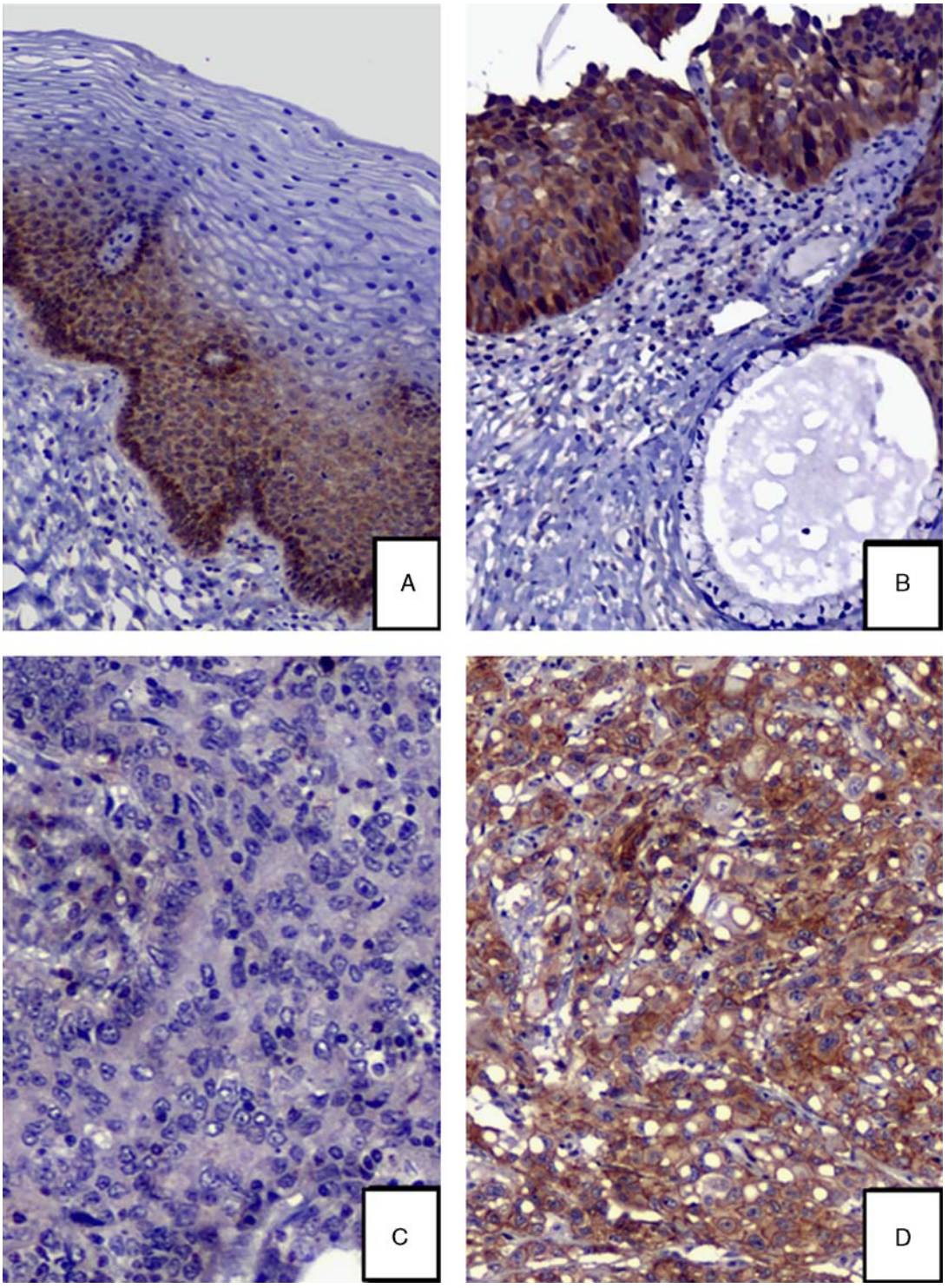

FIG. 4. (A) Microphotograph aspect of EGFR immunohistochemical staining in CIN I (10 ×). (B) EGFR expression in CIN III (10 ×). (C) Microphotograph aspect of low EGFR expression in SCC (20 ×). (D) Aspect of EGFR expression in SCC (20 ×). CIN indicates cervical intraepithelial neoplasia; EGFR, epidermal growth factor receptor; SCC, squamous cervical carcinoma.

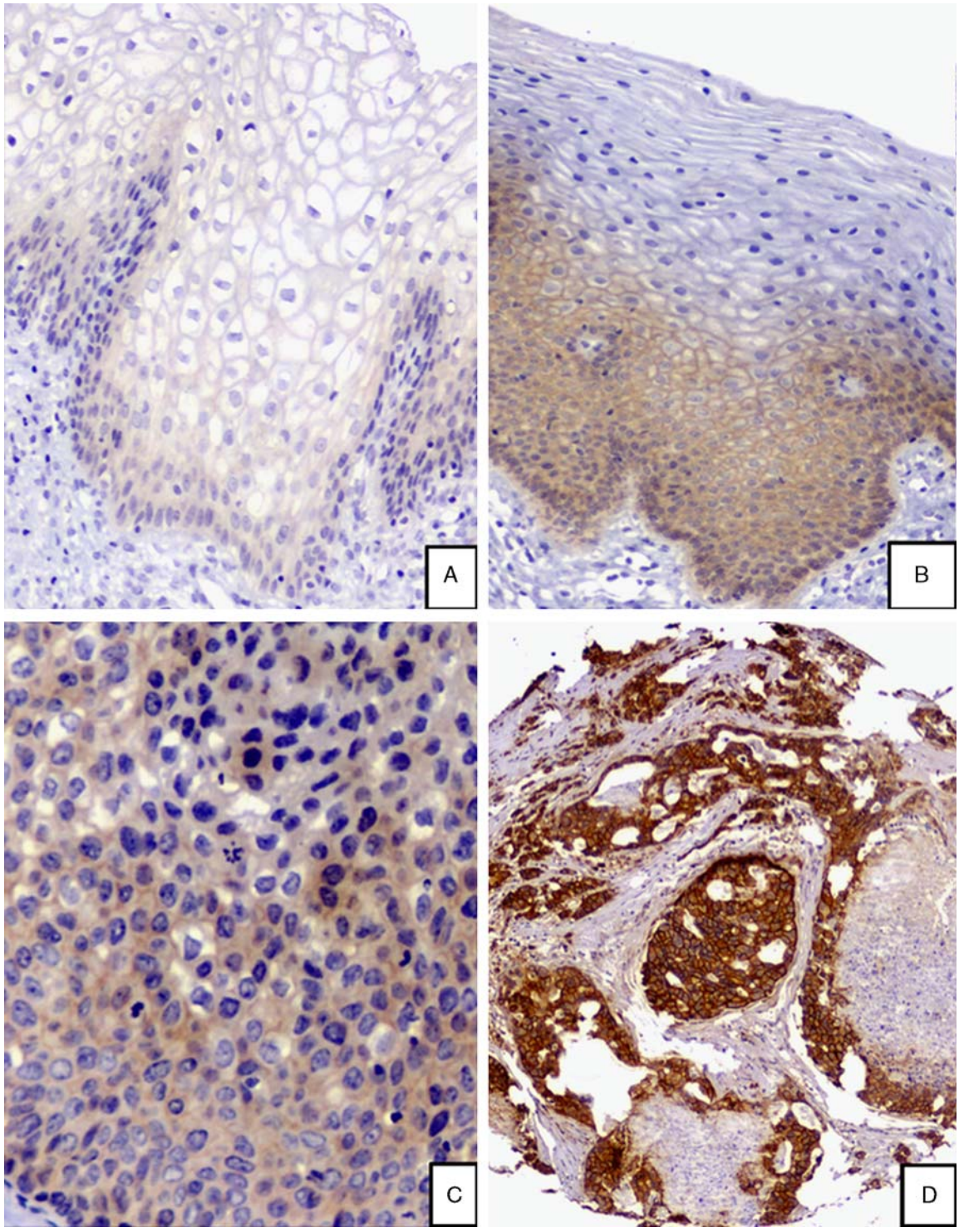

FIG. 5. (A) Microphotograph of ErbB-2 immunohistochemical staining with negative membranous expression in CIN I (10 ×). (B) ErbB-2 expression in CIN II (10 ×). (C) Negative ErbB-2 expression in SCC (20 ×). (D) Microphotograph aspect of positive ErbB-2 expression in SCC (4 ×). CIN indicates cervical intraepithelial neoplasia; SCC, squamous cervical carcinoma.

Lesnikova et al. (61) evaluated ErbB-2 expression in CIN and SCC by immunohistochemistry and chromogenic *in situ* hybridization, reporting no cases of CIN I and II that expressed high levels (3+) versus 2.2% of CIN III cases and 0.7% of SCC cases. Kim et al. (44) also failed to observe ErbB-2 expression in CIN III samples by immunohistochemistry. Other studies have reported immunohistochemical ErbB-2 expression in 0% to 19.8% of SCC cases (51,62–64).

We observed a significant correlation in ErbB-2 expression in the transition from high-grade CIN to SCC. However, ErbB-2 was not expressed in CIN I. During the progression from CIN II to cancer, ErbB-2 was not expressed frequently—2.2% of CIN II, 14% of CIN III, and 16.2% of SCC cases. These findings suggest that ErbB-2 may be relevant for the development and progression of cervical neoplasia in a subset of cases, as we found a statistically significant correlation between ErbB-2 positivity and the transition from CIN to SCC.

Scoring systems for tumor markers in cervical cancer are usually based on the proportion of positive tumor cells and staining intensity (65). Yet, the interpretation of staining intensity not only is highly subjective but can be affected by storage time and variations in protocols and fixation procedures.

Computer-assisted, automated analysis programs help eliminate inherent variability in pathologist-based scores and can increase the sensitivity of protein expression measurements (19–22,66).

We have performed a novel study of CIN and cervical cancer by automated immunohistochemical analysis, demonstrating that the ACIS III system is a sensitive, efficient, and reproducible to quantify COX-2, EGFR, and ErbB-2 expression in cervical tissues. A significant advantage of this method is that it provides a quantitative measure that distinguishes slight differences in staining intensity.

We conclude that COX-2, EGFR, and ErbB-2 expression increases progressively during the progression of CIN to cancer.

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