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Cyclooxygenase-2 (COX-2) Expression in Human Endometrial Carcinoma and Precursor Lesions and its Possible Use in Cancer Chemoprevention and Therapy

A. NASIR[1], D. BOULWARE[2], H.E. KAISER[3], J.M. LANCASTER[1], D. COPPOLA[1], P.V. SMITH[1], A HAKAM[1], S.E. SIEGEL[4] and B. BODEY[4,5]

Departments of [1]Interdisciplinary Oncology-Pathology and [2]Biostatistics, H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida, Tampa, FL; [3]International Society for the Study of Comparative Oncology, Inc. Silver Spring, MD; [4]Childrens Center for Cancer and Blood Diseases, Childrens Hospital Los Angeles, Los Angeles, CA; [5]Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, U.S.A.

Abstract. *In recent years, the design of new antineoplastic agents that can halt the progression of human malignancies with minimal systemic damage has been at the forefront of cancer research, with cyclooxygenase-2 (COX-2) as a major target molecule. With an aim to demonstrate the expression and role of COX-2, the principal putative target of COX-2 inhibitor therapy, in endometrial adenocarcinoma (EACA) and precursor lesions, atypical complex hyperplasia (ACH) and endometrial hyperplasia (EH), an immunohistochemical (IHC) analysis of 22 primary human EACAs and 14 precursor lesions was carried out. Relevant clinicopathological data were tabulated from a random computer-generated sample of 22 primary EACA patients, treated by hysterectomy at our institution. Representative tumor sections including adjacent precursor lesions and normal endometrium (NE) were immunostained with human monoclonal anti-COX-2. Qualitative and semi-quantitative COX-2 IHC staining scores were determined based on the proportion of immunoreactive cells and the intensity of cytoplasmic COX-2 expression. Fisher's exact test and the Wilcoxon Rank Sum test were used for statistical analysis. Mean patient age was 68 years (range 51-93). All 22 EACAs were of endometrioid type, of which ten (45%) were grade I, eight (36%) grade II and four (18%) were grade III. Overall, four out of nine (44%) EHs, four out of five (80%) ACHs, and 18 out of 22 (88%) EACAs were COX-2 positive. The mean COX-2 IHC scores for EH and EACAs were 33 (SD 24.11) and 76 (SD 54.57), respectively (p=0.022). Strong or moderate COX-2 expression was observed in 17 out of 22 (77%) adenocarcinomas as compared to two out of 14 (14%) of the precursor lesions (EH and ACH). The areas of adenomyosis were COX-2 positive, while myometrial smooth muscle and normal fallopian tube tissues stained negative for COX-2. The demonstration of frequent and strong expression of COX-2 in human EACAs supports a possible role for COX-2 inhibitors. Furthermore, an increasing expression of COX-2 from EH to invasive EACAs suggests potential usefulness of COX-2 inhibition to halt the progression of precursor lesions to invasive endometrial cancers.*

Correspondence to: Bela Bodey, MD, D.Sc., University of Southern California, 6820 Remmet Ave. #201, Canoga Park, CA 91303, U.S.A. Tel: +818 716 8612, e-mail: Bodey18@aol.com

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Endometrial cancer, which originates in the inner lining of the uterus, accounts for about 90% of uterine cancers, while uterine sarcoma originates in the myometrium and accounts for less than 10% of the cases (1). About 40,000 American women receive a diagnosis of endometrial cancer each year, making it the fourth most common cancer found in women (1). Adenocarcinoma, which originates in the surface cells of the endometrium, accounts for approximately 90% of cases of endometrial cancer (2). Adenocarcinoma is typically a disease of postmenopausal women and is usually associated with an early onset of symptoms (3).

Molecular targets identified as a result of increasing knowledge of the molecular biological structure and genetic defects have been useful in designing new antineoplastic agents that can halt the progression of human malignancies with minimal systemic damage (4) and cyclooxygenase-2 (COX-2) is emerging as one of the major players among them (5-7). Both cyclooxygenase-1 (COX-1) and COX-2 are catalytic enzymes involved in prostaglandin synthesis (8, 9). Prostaglandin E_2 (PGE_2) functions to promote the primary process of carcinogenesis and its further consolidation and

progression *via* increased cell proliferation, decreased natural killer cell activity, *in situ* immune down-modulation, induction of neoangiogenesis and the elevated expression of antiapoptotic protein Bcl-2 (10). COX enzyme overexpression has been associated with neoplasms at various sites, including the gastrointestinal tract, lung and skin (11). Studies have shown COX-2 overexpression in up to 90% of sporadic colon carcinomas and 40 percent of colonic adenomas (12-15) and in an ever increasing number of other human malignancies, such as lung, head and neck, breast, prostate, brain and pancreas among other sites (16-19). COX-2 is the form of the enzyme cyclooxygenase that is inducible by cytokines, mitogens and growth factors (20), and has emerged as one of the principal targets of the current antineoplastic chemotherapy regimens. In fact, COX-2 inhibitors that have been employed in cancer chemoprevention are now under active investigation for systemic cancer therapy (21).

In order to maximize the benefit of COX-2 inhibitor therapy in the clinical setting, it is important to investigate the role of these markers both in already established human malignancies as well as in their pre-neoplastic lesions.

During the last few years, there has been an emerging interest in the study of COX-2 in human gynecological neoplasms (22) and the clinical role of COX-2 inhibitors in this area is currently a subject of investigation (23). While COX-2 overexpression generally localizes to the adenocarcinoma cells (22, 24), some authors have identified increasing COX-2 presence in the neoplastic cells with increasing FIGO grades of EACAs (20, 25) and have shown an association between COX-2 positivity and shorter disease-free survival (25).

In this study, we focused on the pattern of COX-2 expression in human endometrial adenocarcinoma (EACA) and precursor lesions, atypical complex hyperplasia (ACH) and endometrial hyperplasia (EH). An immuno-histochemical (IHC) analysis of the COX-2 protein in 22 primary human EACAs, in which multiple foci of endometrial hyperplasia and atypical complex hyperplasia were also identified was carried out.

Materials and Methods

Case selection. This study was carried out in line with the research protocol approved by the Institutional Review Board at the H. Lee Moffitt Cancer Center and Research Institute at the University of South Florida, CA, USA. Cases were identified based on a computer search of the surgical pathology archives and pertinent clinicopathological review. A total of 22 consecutive cases of primary invasive EACAs treated by hysterectomy at the HLMCC between 1986 and 1992 were included in the study, on the basis of the following criteria: i) availability of adequate amounts of viable invasive EACA tissue and, as much as possible, the adjacent precursor lesions along with benign non-neoplastic endometrium based on review of the original H&E stained tumor sections by two pathologists with an interest in gynecologic of pathology (AN, AH). ii) The EACA cases had to be treatment-novice, *i.e.* without any prior therapy and iii) availability of a minimum of six years of patient follow-up. Cases that did not meet any of the above criteria were excluded from the study.

Clinicopathological data. In each of the selected cases of EACA, pertinent clinicopathological data were collated and all of the hematoxylin and eosin-stained sections were reviewed for confirmation of histopathological diagnoses including histological grade. The proportions of EACA, EH and non-neoplastic endometrial (NNE) tissue components were also determined for the selected tumor sections.

COX-2 immunohistochemistry. The COX-2 IHC staining protocol was optimized in the core immunohistochemistry laboratory at the University of South Florida, School of Medicine, after testing three anti-COX-2 antibodies (mouse anti-human monoclonal, rabbit anti-human polyclonal and murine polyclonal from Cayman Chemical, USA) under a variety of testing conditions. Based on these results, the primary antibody utilized in this study was an anti-human monoclonal mouse antibody reacting with COX-2 protein in formalin-fixed, paraffin-embedded tissue sections. The immunohistochemical antigen detection was performed using a DAKO autostainer employing 3-micron thick paraffin sections from each of the representative tumor blocks selected. The sections were deparaffinized, rehydrated and subjected to antigen retrieval by using EDTA/Tris buffer at pH 8.0 for 10 minutes in a microwave oven. Endogenous peroxidase was blocked with 3% H_2O_2. The sections were then incubated overnight with a 1:100 dilution of the primary antibody, human monoclonal anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA), according to the COX-2 staining protocol optimized in our laboratory.

The sections were then washed in TBS/Tween and the COX-2 immunoreactivity was detected using a LSAB staining kit and DAB (diaminobenzidene) liquid chromogen (DAKO, Carpenteria, CA, USA). After light counterstaining with modified Mayer's hematoxylin, the sections were cleared, mounted in permount and examined under a light microscope.

Interpretation of immunohistochemical findings. The sections immunostained for COX-2 protein were independently evaluated by both pathologists (AN, AH) without prior knowledge of the clinicopathological data. Immunohistochemical evaluations were made in the most representative and viable areas of the immunostained tumor sections. A semi-quantitative system was used to score the cytoplasmic expression of COX-2, based on the intensity of COX-2 immunostaining (interpreted as 0, 1+, 2+ and 3+ for negative, weak, medium and strong immunostaining respectively) and the proportion of immunostained cells in the EACA tissue and in the areas of EH and NNE present in the same tumor section. A COX-2 immunohistochemical staining score (COX-2 IHC-score) was determined for each component by multiplying the intensity score (1+, 2+ or 3+) with the proportion of COX-2 stained cells (26, 27). In cases with variation in the intensity of COX-2 immunostaining in different areas of the same tumor section, COX-2 IHC score was calculated as follows: COX-2 IHC score= (3 x % of cells staining 3+) + (2 x % of cells staining 2+) + (1 x % of cells staining 1+) (26), as shown in Tables I and II. After independent blinded COX-2 IHC scoring, the semiquantitative COX-2 data from both pathologists were compared. In the case of any discrepancy, a consensus was reached by joint review of the respective immunostained slides.

Table I. *Immunohistochemical expression of COX-2 protein in endometrial adenocarcinoma (N=22).*

Study case #	Patient age	Histopathological tumor type	Histological grade	Percent of total tissue examined	Stained neoplastic cells (%)	COX-2 stain intensity	IHC-score	COX-2 status
1	93	Adenocarcinoma	I	90	80	1	80	P
3	82	Adenocarcinoma	II	95	95	2	190	P
4	78	Adenocarcinoma	III	100	20--40-35	3--2--1	175	P
6	59	Adenocarcinoma	I	20	5	1	5	N
7	62	Adenocarcinoma with squamous metaplasia	I	90	10--20	3--1	50	P
8	71	Adenocarcinoma	II	100	5--40	2--1	50	P
9	67	Adenocarcinoma	I	80	40--50	2--1	130	P
10	52	Adenocarcinoma	I	80	20--30	2--1	70	P
11	69	Adenocarcinoma	I	90	30--70	2--1	130	P
12	59	Adenocarcinoma	II	90	5--40	2--1	50	P
13	61	Adenocarcinoma with squamous metaplasia	II	95	10--10--20	3--2--1	70	P
14	74	Adenocarcinoma	II	95	10--30	2--1	50	P
15	54	Adenocarcinoma	III	80	30--60	2--1	120	P
16	51	Adenocarcinoma	III	100	10--20	2--1	40	P
17	73	Adenocarcinoma	II	100	25--35--25	3--2--1	170	P
18	60	Adenocarcinoma	I	100	15	1	15	N
19	69	Adenocarcinoma	I	85	10	1	10	N
20	67	Adenocarcinoma with squamous metaplasia	II	95	10--20	2--1	40	P
22	54	Adenocarcinoma	I	100	20--40	2--1	80	P
23	81	Adenocarcinoma	III	100	10	1	10	N
24	92	Adenocarcinoma	II	100	10--20--10	3--2--1	80	P
25	77	Adenocarcinoma	I	90	10--40	2--1	60	P
	Av 68; min= 51; max = 93		I=10; II=8; III=4			Mean COX-2 IHC score=	76	

Table II. *Immunohistochemical expression of COX-2 protein in precursors of endometrial adenocarcinoma (N=14).*

Study case #	Patient age	Histopathological diagnosis	Percent of total tissue examined	Stained neoplastic cells (%)	COX-2 stain intensity	IHC-score	COX-2 status
		Atypical complex hyperplasia (ACH): Mean COX-2 IHC score=70					
5	36	Atypical complex hyperplasia	40	50	1	50	P
6	59	Atypical complex hyperplasia	65	100	1	100	P
9	67	Atypical complex hyperplasia	20	80	1	80	P
19	69	Atypical complex hyperplasia	15	0	0	0	N
21	32	Atypical complex hyperplasia	80	20--80	2--1	120	P
		Endometrial hyperplasia (EH): Mean COX-2 IHC score=33					
1	93	Endometrial hyperplasia	10	70	1	70	P
2	88	Endometrial hyperplasia	100	10--40	2--1	60	P
3	82	Endometrial hyperplasia	5	20	1	20	N
12	59	Endometrial hyperplasia	10	20	1	20	N
13	61	Endometrial hyperplasia	5	10	1	10	N
14	74	Endometrial hyperplasia	5	50	1	50	P
15	54	Endometrial hyperplasia	20	5	1	5	N
21	32	Endometrial hyperplasia	20	15	1	15	N
25	77	Endometrial hyperplasia	10	50	1	50	P

Tissue controls. Intestinal smooth muscle cells, vascular endothelial cells and tissue macrophages served as COX-2 positive internal tissue controls. Known COX-2 positive colorectal cancer tissues were also employed as COX-2 positive tissue controls. Negative controls included EACA tissue sections incubated with normal rabbit serum and also morphologically normal fallopian tube sections subjected to COX-2 immunostaining as described above.

Statistical analyses. While comparing the mean COXC-2 IHC scores in EACAs *vs.* endometrial hyperplasia, due to the non-

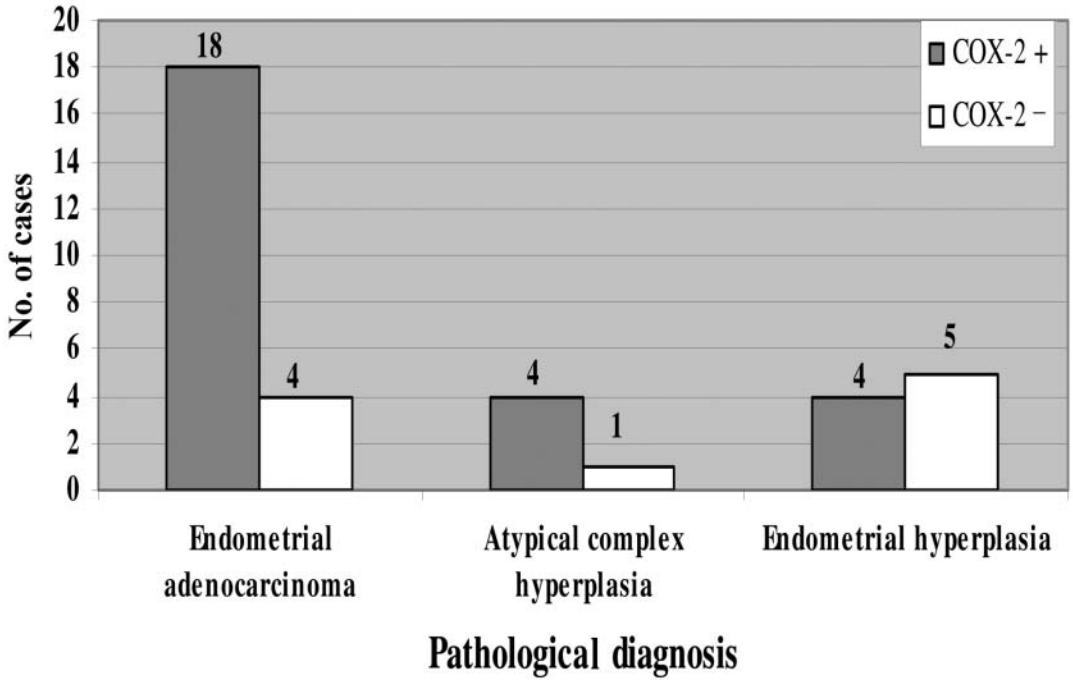

Figure 1. *COX-2 expression in human endometrial adenocarcinoma (N=22) and precursor lesions (N=14).*

normality of the data, the non-parametric test and the Wilcoxon Rank Sum test were used to analyze COX-2 expression in EACAs and their precursors (DB, Biostatistical Unit, HLMCC).

Results

In this series of 22 patients with primary endometrial adenocarcinomas, the mean age was 68 years (range 51-93) (Table I). Nineteen out of 22 (86%) EACAs were pure endometrioid adenocarcinomas, while three (14%) had areas of squamous metaplasia. Regarding the FIGO grading of the endometrioid adenocarcinomas, of the 22 EACAs in this series, ten (45%) were FIGO grade I, eight (36%) FIGO grade II and four (18%) were FIGO grade III (Table I).

COX-2 expression in endometrial adenocarcinoma and precursors. In all of the cases studied, COX-2 immunostaining was localized to the cytoplasm of the cells, both in adenocarcinomas as well as in precursor lesions. In most of the EACAs, a mixed population of COX-2 positive and COX-2 negative neoplastic cells was recognized. The intensity of COX-2 immunostaining was also variable from case to case and within different areas of the same EACA sections. Overall, four out of nine (44%) EHs, four out of five (80%) ACHs and 18 out of 22 (88%) EACAs were COX-2 positive (Figure 1). While the intensity of COX-2 immunostaining varied from mild (1+) to strong (3+) in the EACAs (Table I), all but one case with EH and two cases with ACH had mild (1+) COX-2 expression (Table II).

COX-2 immunohistochemical scores. The mean COX-2 IHC scores in the areas of benign endometrium / EH and EACAs were 33 (SD 24.11) and 76 (SD 54.57) respectively ($p=0.022$) as shown in Figure 2. The intensity of COX-2 immunostaining was moderate to strong in 17 out of 22 (77%) EACAs (Figure 3) as compared to 2 out of 14 (14%) precursor lesions, EH and ACHs (Figure 4). The mean COX-2 immunostaining scores for FIGO grade I, II and III endometrial adenocarcinomas were 63 (N=10), 88 (N=8) and 86 (N=4), respectively.

COX-2 expression in endometrial hyperplasia and non-neoplastic endometrium. In general, the expression of COX-2 in the proliferative phase endometrial glands in non-neoplastic endometrium in our EACA-cases was either weak or absent (Figure 5). While the benign endometrial glands in the areas of adenomyosis showed variable expression of COX-2 protein (Figure 6), myometrial smooth muscle and normal fallopian tube (Figure 7) tissues were COX-2 negative.

Discussion

It is well established that dysregulation and altered functioning of various molecular biological processes and signaling pathways are involved in the development and progression of malignant neoplasms (28). The employment of chemotherapy in combination with radiotherapy has emerged as a strategy of choice in the antineoplastic treatment of advanced human neoplasms (29).

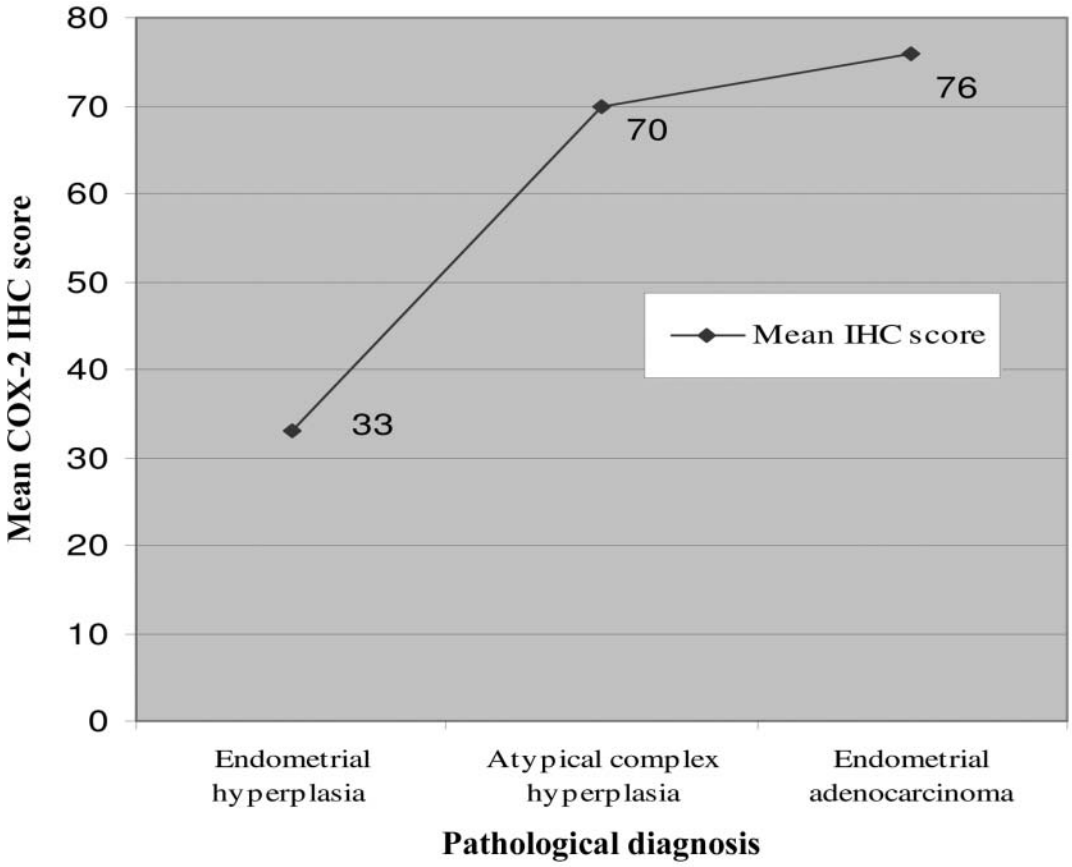

Figure 2. *Mean COX-2 IHC score in endometrial adenocarcinoma, atypical complex hyperplasia and endometrial hyperplasia (N=22, 5 and 9).*

COX-2 has been identified as one of the targets for the non-steroidal anti-inflammatory drugs (NSAIDS) in cancer chemoprevention (30, 31). It has also been linked to carcinogenesis, the maintenance of progressive tumor growth, and facilitation of metastatic tumor spread (18).

Some recent studies have addressed the expression of COX-2 in primary endometrial cancer (22, 24, 25, 32) and its expression has been associated with tumor aggressiveness (25). In the present immunohistochemical study of EACAs and precursor lesions, a high COX-2 positivity rate in human EACA tissues (88%) as compared to EH, ACH and non-neoplastic endometrial tissues evaluated on the same formalin-fixed, paraffin-embedded tissue sections has been demonstrated. These observations are in line with the findings reported in some of the earlier studies (22, 32), while other investigators (24) have found comparable proportions of EACAs (92%) and proliferative endometrium (86%) to express COX-2. In addition, based on the semiquantitative scoring of COX-2 expression in our study, a trend toward an increased expression of COX-2 from endometrial hyperplasia through atypical complex hyperplasia to endometrioid adenocarcinoma was identified. The mean COX-2 immunostaining scores for EH and EACA in our study were 33 and 76 respectively. The overall frequency of COX-2 expression also showed an increase from precursor lesions to EACAs, *i.e.* one out of five (20%) of the cases with foci of ACH and one out of nine (11%) cases with foci of EH showed moderate expression of COX-2 protein, while 17 of 22 (77%) EACAs showed moderate to strong COX-2 expression. If corroborated in additional studies, these findings seem to be of potential clinical relevance in supporting the role of COX-2 inhibition in designing chemoprevention strategies for patients with precursor lesions of endometrial adenocarcinoma. An additional clinical application of our observations is that, since COX-2 is one of the principal targets of COX-2 inhibitor therapy, identifying COX-2 positive EACAs may provide an objective tool to identify a subset of EACAs that may receive a potential therapeutic benefit from COX-2 inhibitor therapy.

The observation that the degree of expression of COX-2 in ACH (mean COX-2 IHC score = 70) is comparable to EACAs (mean COX-2 IHC score = 76) rather than EH (mean COX-2 IHC score 22) suggests that higher expression of COX-2 may be an early event in the neoplastic progression

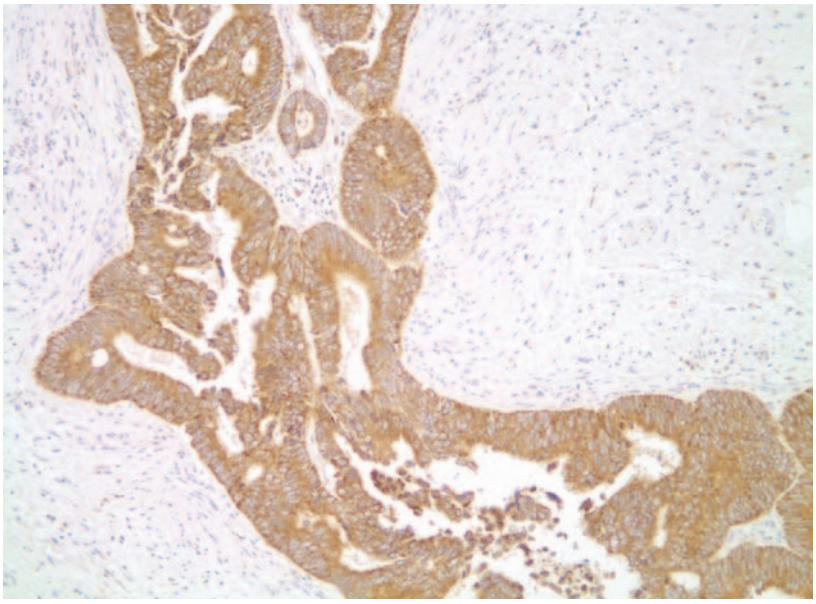

Figure 3. *Atypical glands of endometrial adenocarcinoma showing a strong (3+) expression of COX-2, against unstained tumor stroma (Immunoperoxidase [IMPOX] stain for COX-2. Original magnification x40).*

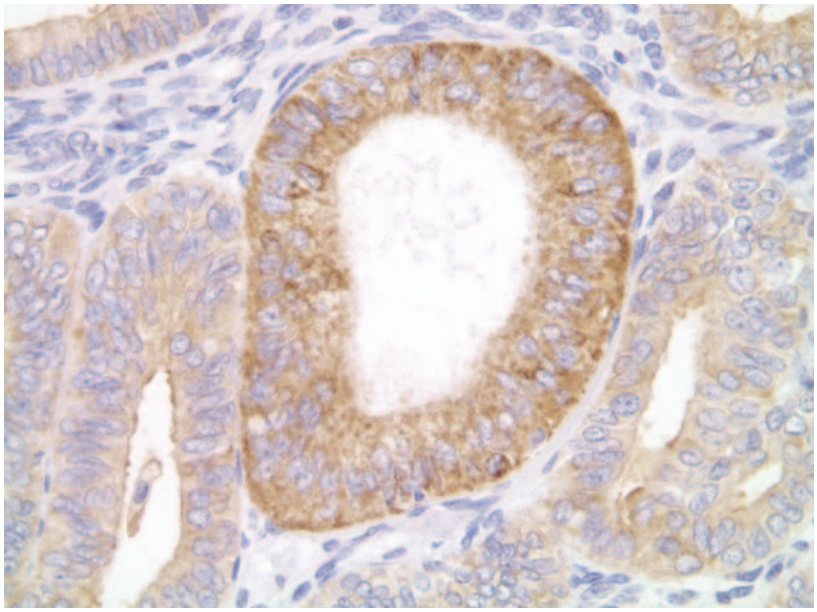

Figure 4. *Some of the glands in atypical complex hyperplasia (ACH) of the endometrium showing moderate (2+) expression of COX-2 (IMPOX stain for COX-2. Original magnification x100).*

of EH to ACH and EACA. Similar observations have been reported in another study (33), in which the expression of COX-2 was found to be higher in cases with atypical endometrial proliferations as compared to normal endometrium and cases with endometritis. While in our experience the degree of COX-2 expression was significantly lower in EH as compared to EACA, a recent study (32), found significantly higher levels of COX-2 both in endometrial cancer and endometrial hyperplasia when compared with normal endometrium. Overall, our findings suggest COX-2 to be over-expressed at some point between progression of EH toward EACA. Further studies on a larger series of cases are warranted to study the expression COX-2 in EACA and precursor lesions. If the trend toward increasing COX-2

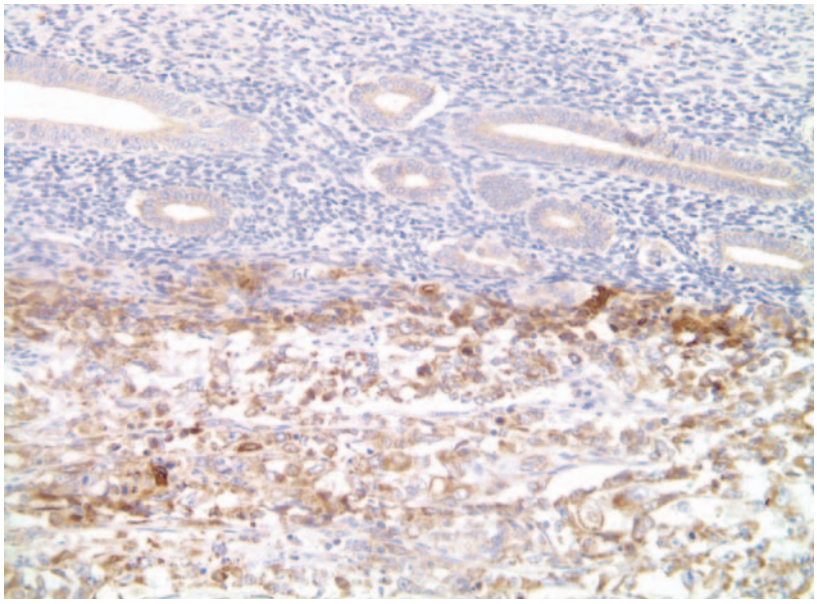

Figure 5. *Non-neoplastic endometrial glands showing absence of cytoplasmic staining for COX-2 (Upper half) as compared to COX-2 positive EACA (Lower half) (IMPOX stain for COX-2. Original magnification x40).*

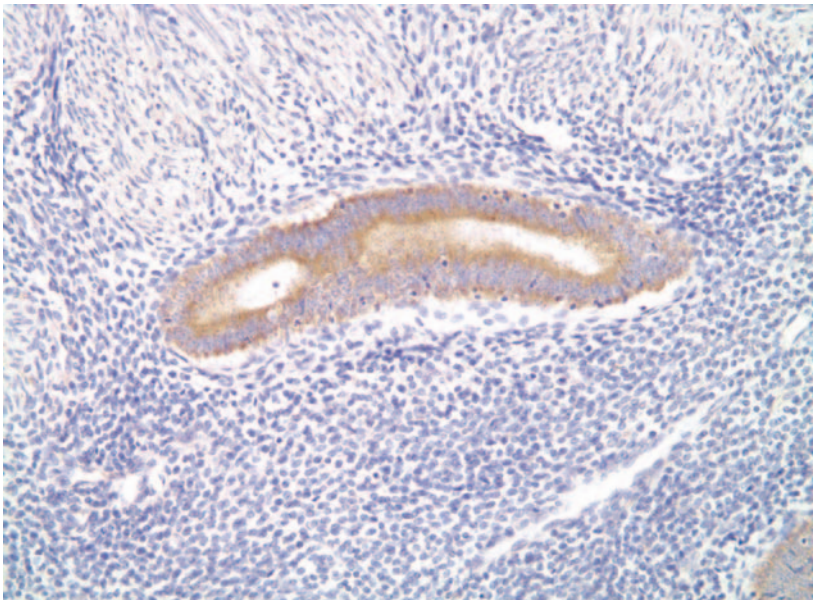

Figure 6. *Benign endometrial glands within a focus of adenomyosis showing moderate (2+) COX-2 staining (IMPOX stain for COX-2. Original magnification x100).*

expression from precursor lesions to invasive EACA remains evident in such studies, COX-2 inhibitors may be considered as potentially useful chemopreventive agents to slow down the progression of EH toward ACH and EACA.

As described by Cao *et al.* (20), well-differentiated EACAs showed minimal COX-2 staining while moderately- and poorly-differentiated EACAs demonstrated the strongest COX-2 expression making a case for a possible role of COX-2 in tumor progression rather than tumor initiation. Similar observations were reported by Ferrandina *et al.* (25). However, such a trend toward higher expression of COX-2 with increasing FIGO grade was not identified in the present cases of EACAs studied. Even though our study had a relatively smaller number of cases in various FIGO grades, there did not

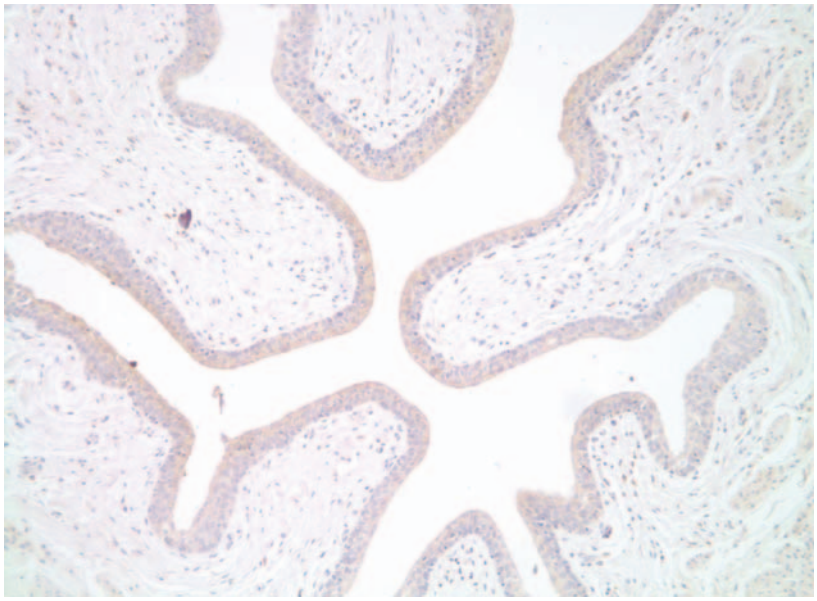

Figure 7. *Section of fallopian tube showing absence of COX-2 in tubal epithelium (COX-2 negative control) (IMPOX stain for COX-2. Original magnification x40).*

appear to be any significant difference in the mean COX-2 immunostaining scores among the various FIGO grades of endometrioid adenocarcinoma. Some of the variation in these data may be explained by the immunohistochemical techniques used for the study of COX-2 expression and the criteria used for the interpretation and scoring of the immunohistochemical results. Additional studies are warranted to investigate the association between COX-2 expression and other pathological findings in human EACA.

COX-2 expression in the coincidental foci of adenomyosis in our cases was variable in intensity. In some cases (Case #s 8 and 24), the intensity of COX-2 immunostaining was found to be 2++. Some preliminary observations have been made on COX-2 overexpression in cases of endometriosis and adenomyosis (34, 35). However, the significance of such variable COX-2 expression in adenomyosis remains unknown at the present time.

Conclusion

The demonstration of frequent and strong expression of COX-2 protein in human EACAs, and a trend toward an increasing expression of COX-2 in ACH and invasive EACAs as compared to EH suggest that the expression of COX-2 is up-regulated following the transition from precancerous to the cancerous (invasive) phase of endometrial carcinoma. These findings also suggest that there might well be a clonal expansion of COX-2 positive, embryonically dedifferentiated, neoplastically transformed endometrial cells and, thus, that expression of COX-2 might well be the "signal" of cancerous transformation in these cells. Furthermore, COX-2 inhibitors may play an important role in the clinical management of endometrial cancer, and may also have a potential chemopreventive role in inhibiting the progression of precursor lesions into invasive endometrial adenocarcinoma. Inhibitors of COX-2 represent another component to be considered when developing the "cocktail" that will lead to the most efficacious treatment of neoplastic disease. Furthermore, routine evaluation of COX-2 status of formalin-fixed, paraffin-embedded human endometrial cancer tissues and precursor lesions may be of potential clinical utility in the effective control of human endometrial cancer and precursor lesions.

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