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ORIGINAL ARTICLE

Estrogen Receptor β as a Prognostic Marker of Tumor Progression in Colorectal Cancer with Familial Adenomatous Polyposis and Sporadic Polyps

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Abstract The incidence of colorectal cancer (CRC) is lower in women than in men, and sex steroids can be considered contributing factors because oral contraception usage and estrogen replacement therapy are associated with decreased risk. Conversely, colorectal polyp development in familial adenomatous polyposis (FAP) begins during puberty. The objectives were to evaluate the relationship between the expression of these hormone receptors and adenoma-carcinoma progression, CRC stage and overall survival. We studied 120 A.C. Camargo Cancer Center patients diagnosed with either FAP-associated or spontaneous adenomatous polyps or CRC to determine the immunohistochemical expression levels of estrogen receptor (ER)-α, ER-β and the progesterone and androgen receptors (480 analyses). The ER-β expression levels differed between the groups: the group with FAP polyps had lower ER-β expression than that of the sporadic polyp group. With transformation of the sporadic polyps to cancer, there was a considerable decrease in ER-β expression (from 90% with strong expression to 80% with absent or weak expression) ($p < 0.001$). The ER-β expression was lower in T3/T4 tumors than in T1/T2 tumors ($p = 0.015$). The 5-year overall survival of CRC patients positively expressing ER-β exceeded that of patients without detectable expression levels (74.8% vs. 44.3%, respectively; $p = 0.035$). There was no significant expression of the androgen or progesterone receptor or ER-α among the groups. Differences in ER-β expression represent a potential mechanism through which estrogen might alter the susceptibility to colon cancer, thereby confirming the possibility of a protective role of estrogen against colorectal carcinogenesis.

Keywords Colorectal cancer · Survival · Estrogen receptor · Familial adenomatous polyposis · Hormonal receptors · Colorectal carcinogenesis

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Introduction

Colorectal cancer (CRC) is a pathology with high incidence and mortality. The highest incidence rates are in Australia, New Zealand, Europe and North America, and the lowest rates are found in Africa and South-Central Asia. These geographic differences appear to be attributable to differences in dietary and environmental factors that are imposed on a background of genetically determined susceptibility [1].

Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by the development of hundreds to thousands of adenomatous polyps in the colon and rectum, and it accounts for 1% of CRC cases. These polyps emerge in the second decade of life, which is during puberty, and increase in size and number during adolescence, which is at the peak of sex steroid production; these findings suggest that sex steroids may act as cofactors in the development of polyps and FAP-related colorectal carcinogenesis [2–6]. Conversely, the incidence of CRC is lower in women than men. Although the cause for this difference is not known, sex steroids may be considered a contributing factor because high parity, early age at first pregnancy, use of oral

contraceptives and estrogen replacement therapy are associated with a decreased risk of sporadic CRC [7].

Giardiello et al. [8] described a patient from the placebo group of a 4-year primary chemoprevention clinical trial of sulindac who developed adenomatous polyps that were later eradicated following the administration of oral contraceptives, suggesting a likely interruption of the adenoma-carcinoma sequence in an FAP patient. However, the possibility of a functional interaction with sex hormone receptors in an APC gene mutation carrier has not been thoroughly investigated.

According to the Women's Health Initiative (WHI) [9, 10], combined (estrogen and progesterone) hormone replacement therapy (HRT) is associated with a decreased risk of CRC (hazard ratio (HR), 0.56), and epidemiological studies have convincingly demonstrated this decreased risk even for the use of estrogen alone [11–14]. Grodstein et al. [15] showed that, in addition to having a lower CRC risk, women who used estrogen had a lower risk of large colorectal adenomas (≥1 cm) compared to that for women who had never used the hormone (HR, 0.74).

Previous studies were also conducted to analyze the presence of hormone receptors in the mucosal epithelium of the normal colon and that of sporadic CRC [16–22]; these studies suggested that the high titer of ER-β observed predominantly in normal tissue is most likely the dominant transcriptional mediator of the estrogen effect that links this effect with a better prognosis [7, 23]. However, no studies have been conducted to evaluate the immunohistochemical expression of estrogen receptor (ER)–α, ER-β and the progesterone and androgen receptors in FAP-associated and sporadic polyps and invasive carcinomas.

The objective of this study was to evaluate the relationship between the expression of these hormone receptors and adenoma-carcinoma progression, CRC clinical and pathological stage and overall survival.

Materials and Methods

Study Subjects

This was a descriptive and analytical cohort study investigating the immunohistochemical expression of ER-α, ER-β and the progesterone and androgen receptors in patients with adenomatous polyps or invasive CRC, with or without a clinical FAP diagnosis. The study was approved by the ethics committee (1453/10).

The sample included all patients with FAP syndrome with cancer between the years 1993–2013 who were treated at the A.C. Camargo Cancer Center and had biological samples stored in the tissue bank; this population is rare because most of these patients are syndromic and underwent preventive CRC surgery with total removal of the colon; these cases determined the sample size. The other groups of patients with adenomatous polyps (FAP and sporadic) and sporadic cancer were defined as randomly treated during the same period. Thus, we evaluated 480 immunohistochemistry exams for ER-α and ER-β, progesterone and androgen receptors for 120 patients, who equally distributed between the following groups:

(A) FAP patients with a histological diagnosis of adenomatous polyps with low-grade dysplasia;
(B) FAP patients with a histological diagnosis of invasive colorectal adenocarcinoma;
(C) patients with sporadic adenomatous polyps with low-grade dysplasia and no clinical diagnosis of FAP or the associated Lynch syndrome; and (D) patients with sporadic invasive colorectal adenocarcinoma and no clinical diagnosis of FAP or the associated Lynch syndrome.

The follow-up routine for patients with colorectal tumors treated at the institution included a clinical visit every three months during the first two years; the routine included laboratory tests, tumor marker evaluation, chest X-ray and abdominal ultrasound or cross-sectional imaging (tomography), alternately. From the third to the fifth year, this routine examination was performed every six months; afterward, this procedure was conducted annually. Proctosigmoidoscopy was repeated annually in FAP patients who underwent surgery.

Antibodies, Construction and Immunohistochemical Analysis

The immunohistochemistry was performed using two slides of the tissue microarray (TMA) blocks for each antibody. TMA sections from the array block were stained with the following primary antibodies: anti-ER (clone SPL Dako), anti-ER-β (clone 14C8, Genetex), anti-progesterone receptor (clone PgR 636, Dako) and anti-androgen receptor (clone AR 441, Neomarkes). Four-micrometer-thick sections were cut from the array block and were deparaffinized with xylene and dehydrated through a graded alcohol series. Microwave antigen retrieval was used for all of the antibodies: the slides were placed in 10 mM citrate buffer (pH 6) for 15 min. A standard peroxidase-conjugated streptavidin-biotin method was used to detect the staining reaction (Advance, HRP Link, DAKO, Carpinteria, USA). External positive control tissues included breast and prostate samples from normal tissue that were positive for the studied antibodies. For negative controls, the primary antibodies were omitted and substituted with normal serum antigen.

A scoring system was applied based on the intensity as recommended by Rüschoff et al. [24] using an optical microscope with objective magnification. The intensity was classified as strong when nuclear staining was observed at 5X

magnification, moderate for staining observed at 10X and weak for staining that was only observed at 40X (Fig. 1).

The scoring was conducted independently by 2 pathologists: M.D.B. and M.M. The preparation for the immunohistochemistry and the scoring were conducted in a manner blinded to the other case characteristics. Discordant results were resolved by an additional joint review of the relevant sample.

Statistical Analysis

For statistical analysis, the software Statistical Package for Social Science (SPSS) version 20 was used. Survival curves were estimated using the Kaplan-Meier product-limit method, and significant differences between the survival curves were determined using the log-rank test. All variables associated with survival with $p < 0.20$ in univariate analysis were included in multivariate analysis using a Cox proportional hazard regression model. Correlation coefficients between all findings were estimated using Pearson's chi-squared test or a chi-squared two-tailed corrected test (Fisher's exact test) as indicated. The results were considered statistically significant at $p < 0.05$.

Results

Clinical and Histopathological Results

For the 120 assessed patients, the median age was 49 years (19–80 years), 48% were males, and 52% were females. For patients with invasive cancer, the most frequent clinical complaint was bleeding (32% of cases). The most frequent site of primary tumor was the colon (70%), followed by the rectum (30%). Moderately differentiated adenocarcinoma represented 56.7% of the cases. In FAP patients, the median age for disease diagnosis was 31 years, and that for cancer diagnosis was 38 years. The median follow-up time of cancer patients in the study was 61 (4–239) months. Table 1 shows clinical and pathological profiles according to the expression of ER-β.

Immunohistochemical Results

ER-β expression was significantly different between groups (Fig. 2). Compared with the sporadic polyp group, the FAP polyp group had lower expression levels of these receptors. In the sporadic group, CRC transformation was accompanied by a considerable decrease in the high ER-β expression observed in sporadic polyps, where 90% of the samples strongly expressed ER-β and 100% exhibited some degree of expression. This finding contrasts with that for the sporadic cancer group, where 80% exhibited a low or complete lack of ER-β expression (40% negative, 40% weak expression, 20% moderate expression and 0% strong expression; $p < 0.001$).

Patients with T3/T4 tumors demonstrated a significant absence in ER-β expression compared to that of patients with T1/T2 tumors (T1/T2: 5.8% negative, 94.2% positive vs. T3/T4: 37.2% negative, 62.8% positive; $p = 0.015$) (Fig. 3). There was no significant difference in expression with regard to lymph node status (N0: 25% negative, 75% positive vs. N+: 33.3% negative, 66.7% positive; $p = 0.483$) and metastatic status (M0: 30.2% negative, 69.8% positive vs. M+: 14.2% negative, 85.8% positive; $p = 0.380$).

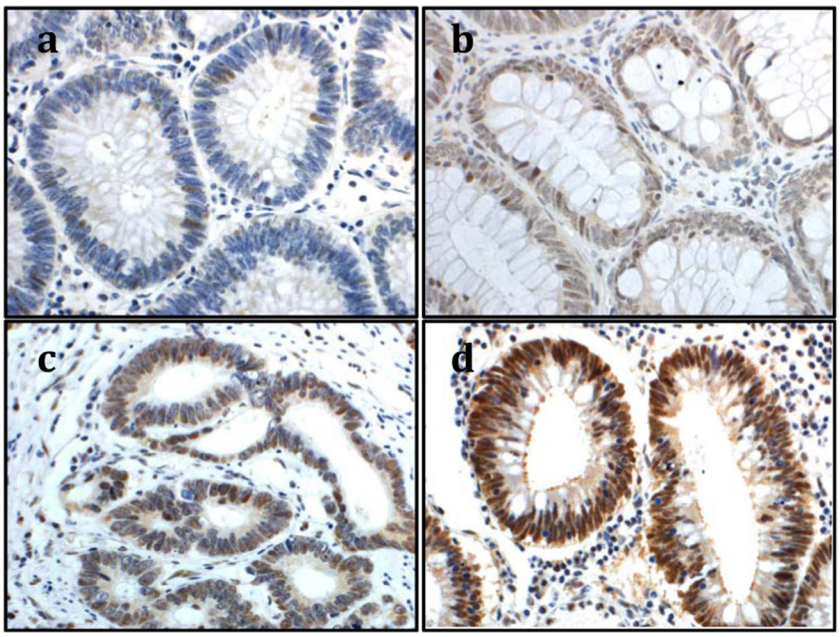

Fig. 1 Photomicrograph of a TMA sample showing the immunohistochemical (IHC) differences found using anti-ER-β (14C8 antibody) in adenomatous polyps. **a** – Absence of ER-β expression. (×40). **b** – Weak ER-β expression. (×40). **c** - Moderate ER-β expression. (×20). **d** - Strong ER-β expression. (×40)

Table 1 Clinical and pathological profiles according to the expression of ER-β

Group*	n	Expression of ER-β				
		Absence	Weak	Moderate	Strong	p
Gender						
Male	58	15.5% (9)	36.2% (21)	15.5% (9)	32.8% (19)	0.420
Female	62	24.2% (15)	24.2% (15)	19.4% (12)	32.3% (20)	
Stage T						
T1/T2	17	5.8% (1)	41.2% (7)	41.2% (7)	11.8% (2)	0.087
T3/T4	43	37.2% (16)	34.9% (15)	20.9% (9)	7% (3)	
Stage N						
N0	36	25% (9)	36.1% (13)	27.8% (10)	11.1% (4)	0.744
N+	24	33.3% (8)	37.5% (9)	25% (6)	4.2% (1)	
Stage M						
M0	53	30.2% (16)	35.8% (19)	26.4% (14)	7.5% (4)	0.805
M+	7	14.2% (1)	42.9% (3)	28.6% (2)	14.3% (1)	
Location of the tumor						
Colon	42	21.4% (9)	40.5% (17)	26.2% (11)	11.9% (5)	0.167
High rectal	18	44.4% (8)	27.8% (5)	27.8% (5)	0%	
Histologic Grading and Differentiation						
Low grade (G1)	23	21.7% (5)	43.5% (10)	26.1% (6)	8.7% (2)	0.788
High grade (G2/G3)	37	32.4% (12)	32.4% (12)	27.0% (10)	8.1% (3)	
Vascular Embolization						
No	52	25% (13)	38.5% (20)	28.8% (15)	7.7% (4)	0.430
Yes	8	50% (4)	25% (2)	12.5% (1)	12.5% (1)	
Lymphatic Embolization						
No	45	31.1% (14)	35.6% (16)	24.4% (11)	8.9% (4)	0.813
Yes	15	20% (3)	40% (6)	33.3% (5)	6.7% (1)	
Perineural Embolization						
No	43	27.9% (12)	37.2% (16)	23.3% (10)	11.6% (5)	0.447
Yes	17	29.4% (5)	35.3% (6)	35.3% (6)	0%	
Total Group						
FAP Polyp	30	23.3% (7)	40% (12)	13.3%(4)	23.3%(7)	< 0.001
FAP Cancer	30	16.7% (5)	33.3% (10)	33.3%(10)	16.7%(5)	
Sporadic Polyp	30	0%	6.7% (2)	3.3% (1)	90% (27)	
Sporadic Cancer	30	40% (12)	40% (12)	20% (6)	0%	

*Cancer Group (n = 60) / Polyp Group (n = 60) / Total Group (120)

There was no expression of androgen and progesterone receptors among the groups, as well as no significant expression of ER-α, which was present in only 3 individuals of the sporadic polyps group.

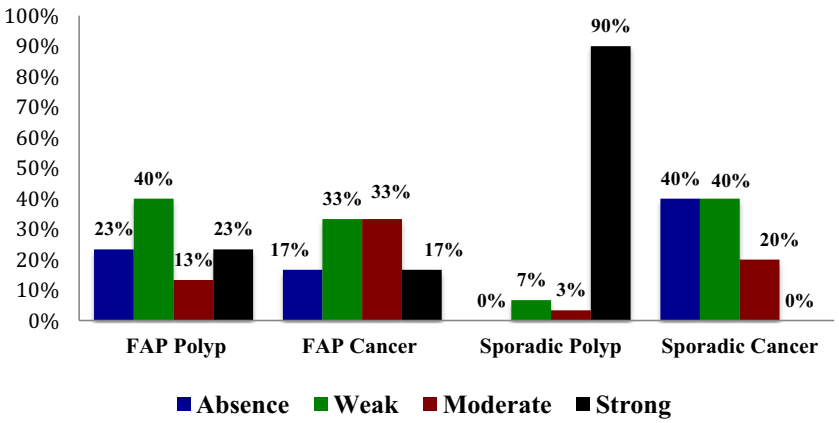

Fig. 2 Immunohistochemical expression of ER-β (n = 120) in the different groups. $p < 0.001$

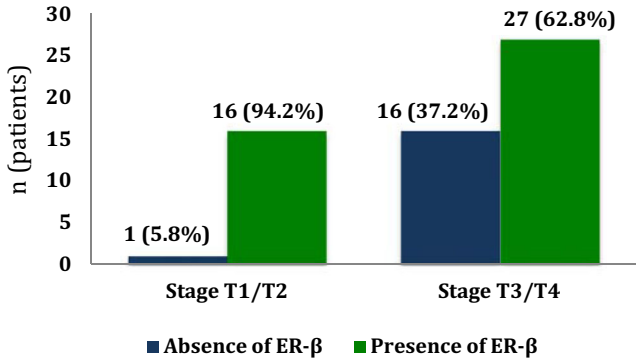

Fig. 3 Immunohistochemical expression of ER-β with regard to T stage (TNM). $p = 0.015$

Overall Survival Results

The 5-year overall survival of all patients with CRC was 65.3% (Table 2). The highest survival rate was observed in patients showing some degree of ER-β expression compared to that in patients with no expression (74.8% vs. 44.3%, $p = 0.035$) (Fig. 4). Univariate analysis showed significantly better overall survival for the following characteristics: positive for ER-β, younger than 50 years of age, absence of lymphatic embolization and Clinical Stage I/II (Table 2).

Multivariate analysis was performed for the variables clinical stage, lymphatic embolization, expression of ER-β and the presence of FAP syndrome to determine the characteristics with a greater risk of death ($p < 0.2$, log rank). From the multivariate Cox regression model, clinical stage III/IV (HR 2.9, 95% CI = 1.15–7.43, $p = 0.023$), presence of lymphatic embolization (HR 2.6, 95% CI = 1.03–6.78, $p = 0.043$) and absence of ER expression (HR 3, 95% CI = 1.24–7.46, $p = 0.015$) showed a significantly higher risk of death (Table 3).

Table 2 Univariate analysis of prognostic factors for 5-year overall survival (OS) in cancer patients

Variable	n	5-year OS %	p
ER-β			
Negative	17	44.3%	0.035
Positive	43	74.8%	
Age			
< 50 years	28	74.5%	0.040
> 50 years	32	57.7%	
Gender			
Male	28	61%	0.716
Female	32	68.5%	
Location of the tumor			
Colon	42	69%	0.716
High rectal	18	68.5%	
FAP/Sporadic Cancer			
FAP	30	69.9%	0.095
Sporadic	30	60%	
Clinical Stage			
Stage I/II	35	81.5%	0.002
Stage III/IV	25	36.1%	
Vascular Embolization			
No	52	73.9%	0.224
Yes	8	33.3%	
Lymphatic Embolization			
No	45	73.9%	0.022
Yes	15	53.8%	
Perineural Embolization			
No	43	70.8%	0.641
Yes	17	63%	
Histologic Grading and Differentiation			
Low grade (G1)	22	70.7%	0.705
High grade (G2/G3)	38	61.6%	

Discussion

In this study, analysis of the expression of the studied hormonal receptors (androgen, progesterone and estrogen α and β) showed dominant expression levels of ER-β in adenomatous polyps, and these levels varied in sporadic CRC and FAP patients. This fact, associated with the underexpression or absence of expression of the other sex hormone receptors, supports the hypothesis that estrogen signaling through ER-β plays a role in colorectal carcinogenesis. Biological evidence supports this association and has been observed in other studies, where an overexpression of ER-β was detected in normal colonic mucosa compared to that in sporadic CRC [15–21]; these results also apply to our findings in adenomas.

We observed lower ER-β expression levels in FAP polyps than in sporadic polyps. This finding, when associated with studies in an experimental model of APC-deficient mice,

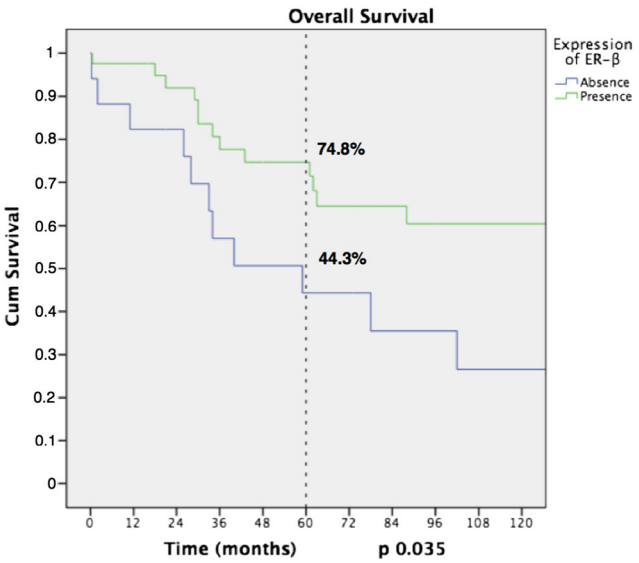

Fig. 4 Overall survival of CRC patients with regard to ER-β expression

Table 3 Multivariate analysis of prognostic factors for overall survival (OS) in cancer patients

Variable	HR*	p	95% CI**
Clinical stage (TNM)			
Stage I/II	1	0.023	1.15–7.43
Stage III/IV	2.9		
Lymphatic Embolization			
No	1	0.043	1.03–6.78
Yes	2.6		
Expression of ER-β			
Positive	1	0.015	1.24–7.46
Negative	3		
Age			
< 50	1	0.215	0.69–4.94
> 50	1.8		
FAP/Sporadic Cancer			
- Sporadic	1	0.464	0.46–5.35
- FAP	1.5		

* HR (hazard ratio) / **95% CI (95% confidence interval)

which had a loss of ER expression in colorectal tumors [25], may suggest that these receptors act as inhibitory modifiers of APC-dependent colon tumorigenesis. Cho et al. [26] demonstrated that oophorectomy resulted in a significant increase in the number of intestinal adenomas in mice using an animal model of APC-dependent CRC. Currently, little is known about estrogen signaling in FAP patients, but these findings favor a new line of investigation regarding whether estrogen actions can contribute to or cooperate with the tumor suppressive function of the APC gene. A double-blind, randomized, 4-year primary chemoprevention study investigated sulindac usage in genotypically affected individuals (APC gene mutation) who were initially not phenotypically affected; the results for the placebo group showed complete eradication of polyps with the occasional administration of an oral contraceptive to a patient who had developed polyps [8]. That same study demonstrated no changes in prostaglandin levels in the colonic mucosa, which makes anti-inflammatory drug administration an unlikely cause of this effect. Flexible proctosigmoidoscopy was performed for follow-up every four months for 48 months, and a recurrence and increase in the prevalence of polyps was observed after suspension of the oral contraceptive.

We observed significantly lower ER-β expression levels in the CRC groups than in the sporadic polyp group, with an increased loss of expression related to more advanced T stages (TNM [27]) (T3/T4 tumors vs. T1/T2 tumors). This finding might explain why we did not observe lower expression in the FAP cancer group compared to that in the FAP polyp group because the FAP cancer group was 56.7% T1/T2 patients, while the sporadic cancer group mainly had T3/T4 patients (Fig. 3). Other studies [28, 29] also reported a relationship between the loss of ER-β expression in CRC with Dukes' advanced stage and a higher degree of tumor differentiation. Therefore, in the groups with polyps (FAP and sporadic), we only included patients with low-grade dysplasia because polyps with high-grade dysplasia could have a similar behavior to the cancer groups. These data indicate that the presence of ER-β has an important role in colorectal carcinogenesis as a marker of tumor progression and could explain the protective effect of estrogen against the development of CRC, as well as the worsened prognosis for patients with tumors lacking ER-β expression.

Among the numerous types of cancer, breast cancer has a model of hormonal carcinogenesis that is the most thoroughly described and understood, which has allowed the discovery of important prognostic markers and the establishment of anti-hormone therapies [30]. Subsequently, there has been an effort to understand the mechanism through which estrogen and other steroid hormones could act in carcinogenesis pathways. Estrogens control a wide range of vital physiological processes that are chiefly mediated by the activities of ER-α and ER-β, which are closely related to the nuclear receptors of ligand-dependent transcription factors and are at the promoters of estrogen-sensitive genes [31–33]. ERs may act as transcription cofactors through their interaction with other DNA-binding proteins, and they also mediate non-specific estrogen-induced physiological effects through genomic mechanisms of action [34, 35].

It has been reported that the administration of oral estrogen during HRT has reduced the risk of CRC development by approximately 44% and lessens the recurrence of neoplastic polyps after surgical removal of colon tumors [9, 10, 36, 37]. Thus, to evaluate one effect of the presence of ER-β expression, we assessed overall survival in terms of certain clinical pathological variables and the expression of ER-β. We observed a better overall survival in individuals with CRC associated with positive expression of ER-β ($p = 0.035$), and the loss of ER-β expression remained an independent prognostic factor of a higher risk of death (HR 3, 95% CI = 1.24–7.46, $p = 0.015$) (Tables 2 and 3).

In summary, our study suggests that ER-β expression might mediate the chemopreventive effects of estrogens in the development of polyps and CRC. Based on these findings, new questions arise, and further studies might be useful in the search for chemopreventive treatments for patients with FAP. Moreover, these data also suggest the need for greater surveillance and screening of polyps and CRC in individuals who receive anti-estrogen hormone treatment (tamoxifen/anastrozole) due to other diseases, as well as in patients who have undergone oophorectomy, are nulliparous, are in early menopause or have risk of hereditary breast or CRC syndrome. As in breast cancer, in CRC, the level of hormone receptor expression, ER-β in particular, is related to patient prognosis, supporting the role of ER-β as an important marker

of tumor progression and a possible target for chemoprevention in patients at risk for CRC.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that there is no conflict of interest.

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