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• COLORECTAL CANCER •

Expression of estrogen receptor b in human colorectal cancer

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Abstract

AIM: To determine the expression of estrogen receptor (ER)β in Chinese colorectal carcinoma (CRC) patients.

METHODS: ERβ expression in CRC was investigated by immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections from 40 CRCs, 10 colonic adenomas, and 10 normal colon mucosa biopsies. The percentage of positive cells was recorded, mRNA expression of ERα and ERβ in 12 CRC tissues and paired normal colon tissues were detected by RT-PCR.

RESULTS: Positive ER immunoreactivity was present in part of normal epithelium of biopsy (2/10), adenomas (3/10), and the sections of CRC tissue, most of them were nuclear positive. In CRCs, nuclear ERβ immunoreactivity was detected in over 10% of the cancer cells in 57.5% of the cases and was always associated with cytoplasmic immunoreactivity. There was no statistical significance between ERβ positive and negative groups in regard to depth of invasion and nodal metastases. Of the 12 CRC tissues and paired normal colon tissues, the expression rate of ERα mRNA in CRC tissue and corresponding normal colon tissue was 25% and 16.6%, respectively. ERβ mRNA was expressed in 83.3% CRC tissue and 91.7% paired normal colon tissue, respectively. There was no significant difference in ERβ mRNA level between CRC tissues and paired normal colon tissues.

CONCLUSION: A large number of CRCs are positive for ERβ, which can also be detected in normal colonic epithelia. There is a different localization of ERβ immunoreactivity among normal colon mucosae, adenomas and CRCs. ERα and ERβ mRNA can be detected both in CRC tissue and in corresponding normal colon tissue. A post-transcriptional mechanism may account for the decrease of ERβ protein expression in CRC tissues.

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INTRODUCTION

Epidemiological data have shown that the risk of colorectal cancer is reduced among postmenopausal hormone users, compared with those who have never used these hormones. Animal models showed that male rats had a higher risk developing colon cancer compared with their female counterparts when exposed to dimethylhydrazine, an experimental carcinogen. The results indicated that 17β-oestradiol (E_2) treatment could significantly reduce the frequency of dimethylhydrazine-induced large intestinal tumors in rats[1-3]. These evidences suggest that estrogen maybe involved in the growth of colonic tumors. Estrogen receptor locates at the cellular nuclei of target tissues, estrogen molecules diffuse into cytoplasm, and bind to estrogen receptors, then modulate gene expression by interaction with promoter response elements or other transcription factors. The estrogen receptor discovered in 1986 is named ERα, and another ER subtype identified in 1997 is called ERβ. ERβ protein contains 485 amino acids, with a molecular weight of 54.2 Kda. The DNA binding domain (DBD) contains a two-zinc finger structure which plays an important role in receptor dimerization and in binding of receptors to specific DNA sequence. The DBDs of ERα and ERβ are highly homologous[4]. Up to now, several ERβ isoforms have been identified such as ERβ1, ERβ2, ERβ3, ERβ4, ERβ5, *etc*[5]. However, the physiological significance of these ERβ isoforms is still unknown. Meanwhile, published data about the expression of ERα and ERβ in colon cancer tissues were often controversial[6-20]. Therefore it is reasonable to reevaluate ER status and hormonal modulation of cell growth in colon cancer. In this study, immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) techniques were used to explore the precise mechanism of hormonal modulation of colon cancer.

MATERIALS AND METHODS

Patients and tissues

The study was performed in tissue sections of CRC from 40 patients who underwent surgical resection of colorectal cancer in the Department of Surgery of Renmin Hospital, Wuhan University from June 1998 to December 2000. The age of the patients ranged from 38 to 78 years (average age 65 years). Ten sections of colonic adenoma were studied, and 10 sections of normal colonic mucosa biopsy were used as control. Information about depth of invasion and nodal metastases was obtained from a review of the pathology reports. Fresh tumor tissues and corresponding normal colon tissue were obtained from 12 patients who underwent surgical resection of colon cancer in the Department of Surgery of Affiliated Hospital of Wujing Medical College and Tianjin First Central Hospital from June 2001 to December 2002. The patients were comprised of 8 men and 4 women aged 49-79 years (mean 63.3 years). The tumorous and paired normal tissues were divided into two parts. One part was fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin for pathological diagnosis. The other part was frozen in liquid nitrogen and stored at -80 ℃ until RNA was extracted. ERα and ERβ mRNA were detected by RT-PCR.

Immunohistochemistry

Rabbit anti-rat or human ERβ polyclonal antibody was purchased from Santa Cruz, USA. S-P kit and DAB kit were purchased from Fuzhou Maixin Biotechnology Company.

Formalin-fixed and paraffin-embedded tissue sections from 40 CRCs, 10 colonic adenomas, and 10 normal colonic mucosa biopsies were immunostained by SP technique with the following procedures. The slides were washed in 0.01 M phosphate-buffered saline (PBS). Endogenous peroxidase was

blocked by 0.3% H_2O_2 for 25 minutes, followed by incubation in normal goat serum for 15 minutes at room temperature. Then the slides were incubated with a 1:75 dilution of the primary ERβ polyclonal antibody for 2 hours at room temperature. After that the slides were washed with a reagent (biotinylated anti-immunoglobulin) for 20 minutes at room temperature. After rinsed in PBS, the slides were incubated with the peroxidase-conjugated streptavindin label for 20 minutes at room temperature, and incubated with diaminobenzidine and H_2O_2 for 5 minutes. Finally the sections were counterstained with hematoxylin.

RT-PCR amplification

Total RNA was isolated with TRIZOL reagent (Life Technologies, USA), and quantified by spectrometry (λ260 nm). Only those RNA preparations with 260/280>1.7 were used in this study.

Reverse transcription was performed using a reverse transcription system (revertaid™ first strand cDNA synthesis kit, MBI). RT of RNA was performed in a final volume of 20 μl containing 5×first strand buffer (containing 1 mM Tris-HCl. pH8.3, 1.5 mM KCl and 60 μM $MgCl_2$), 25 μM dNTP mixture, 200 pM random primer, 100 units of Moloney murine leukemia virus reverse transcriptase, 2 μg total RNA. Then DEPC treated water was added to 20 μl. RT reaction procedure was as follows: at 70 ℃ for 1 min→at 37 ℃ for 5 min→at 42 ℃ for 60 min→ at 98 ℃ for 5 min. ERα, ERβ and β-actin were amplified using several pairs of appropriate oligonucleo tide primers as follows: ERα (530 bp): (sense)5' -ATGTGGGAGAGGAT GAGG AG-3' , (antisense) 5' -AACCGAGATGATGTAGCCAGCAGC-3' . ERβ (259 bp): (sense) 5' -TAGG GTCCATGGCCAGTTAT-3' , (antisense) 5' -GGGAGCCACACT TC ACCAT-3' . β-actin (control) (540 bp): (sense) 5' -GTGGG GCGCC CCAGG CAC CA-3' , (antisense) 5' -CTTCC TTAAT GTCAC GCACG ATTTC-3' (Figure 1).

PCR was performed in a final volume of 50 μl containing 4 μl 10×pc buffer, 2.5 U recombinant Taq DNA polymerase (Taraka, Japan), 0.1 mM $MgCl_2$, 100 μM dNTP mixture, and 50 pM of each primer. PCR was performed for 40 cycles (denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extention at 72 ℃ for 1.5 min). The PCR condition for inter control β-actin was 35 cycles (denaturation at 94 ℃ for 45 seconds, annealing at 60 ℃ for 45 seconds, and extention at 72 ℃ for 45 seconds). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining and UV illumination.

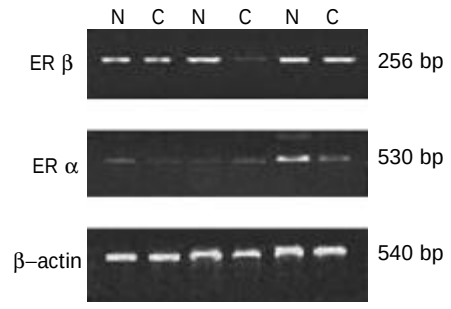

Figure 1 mRNA expression of ERα and ERβ in paired representative tissues from cancer and adjacent normal mucosa. C: cancer, N: normal mucosa. RT-PCR result of β-actin was used to show equal loading.

Statistical analysis

The association between expression of ERα and ERβ, the significance of ERα and ERβ in groups dichotomized according to clinical and histopathologic characteristics of the patients were compared and assessed by Chi-square test and Student' s *t*-test. A *P* value <0.05 was considered significant.

RESULTS

Expression of ERa and ERb protein

Using immunoperoxidase technique, ERβ immunoreactivity was detected in or near the nuclei of normal colonic mucosa in the same sections of carcinoma. ER immunoreactivity was present in some of normal epithelia (2/10), and adenomas (3/10). Nuclear immunoreactivity was consistently found in part of normal colonic mucosa, and in all areas of the crypt epithelium, and most abundant at the bottom (Figures 2A,2B). One section of rectal tubular adenocarcinoma showed strong positive nuclear and cytoplasmic staining of ERβ (Figure 2C). A few stromal cells, smooth muscle cells and vascular endothelial cells were also positive (Figure 2D). In CRCs, nuclear ERβ immunoreactivity was associated with cytoplasmic immunoreactivity. Some sections showed only cytoplasmic staining of ERβ (Figure 2E), Positive ERβ was detected in more than 10% of the cancer cells in 57.5% of the CRC cases (Figure 3).

Three of the 12 randomly selected cases stained with anti-ERα antibody showed positive. None of the 10 normal colonic mucosa biopsies was stained positive with anti-ERα antibody. There were no statistically significant differences between positive and negative ERβ groups in regard to the depth of invasion, and nodal metastases (Tables 1-2).

Table 1 Expression of ERβ and ERα in CRCs, colonic adenomas and normal colonic mucosa

Group	*n*	ERα positive (%)	*n*	ERβ positive (%)
Normal colon mucosa	10	0 (0%)	10	2 (20%)
CRCs	12	3 (25%)	40	23 (57.5%)
Colonic adenoma			10	3 (30%)

Table 2 Clinicopathological characteristics of patitents with CRCs and their association with ERβ expression

Category	*n*	ERβ negativity	ERβ positivity(%)	*P* value
Age (years)				>0.05
<50	4	2	2 (50.0)	
⩾50	36	15	21 (58.3)	
Sex				>0.05
Male	23	9	14 (60.9)	
Female	17	8	9 (52.9)	
Lymph node metastasis				>0.05
0	18	7	11 (61.1)	
⩾1	22	10	12 (54.5)	
Duke's type				>0.05
A	15	5	10 (66.7)	
B	10	3	7 (70.0)	
C	7	3	4 (57.1)	
D	3	1	2 (66.7)	
Histological grading				>0.05
Well -differentiated	15	5	10 (66.7)	
Moderately- differentiated	15	7	8 (53.3)	
Poorly- differentiated	10	5	5 (50.5)	

Table 3 Expression of ERα mRNA in CRC tissue and adjacent normal mucosa tissue

Tissue type	*n*	+	Positive rate (%)
CRCs	12	3	25%
Normal tissue	12	2	16.6%

Table 4 Expression of ERβ mRNA in CRC tissue and adjacent normal mucosa tissue

Tissue type	*n*	+	Expression rate (%)	Level of ERβmRNA	*P*
CRCs	12	10	83.3%	91.15±3.56	>0.05
Normal tissue	12	11	91.7%	95.38±2.79	

ERa and ERb mRNA expression

Table 3 and Table 4 show that the expression of ERα mRNA in CRC tissue and corresponding normal colon tissue was 25% and 16.6%, respectively. ERβ mRNA was predominantly expressed in CRC tissue and paired normal colon tissue, the positive rate was 83.3% and 91.7%, respectively. There was no statistically significant difference.

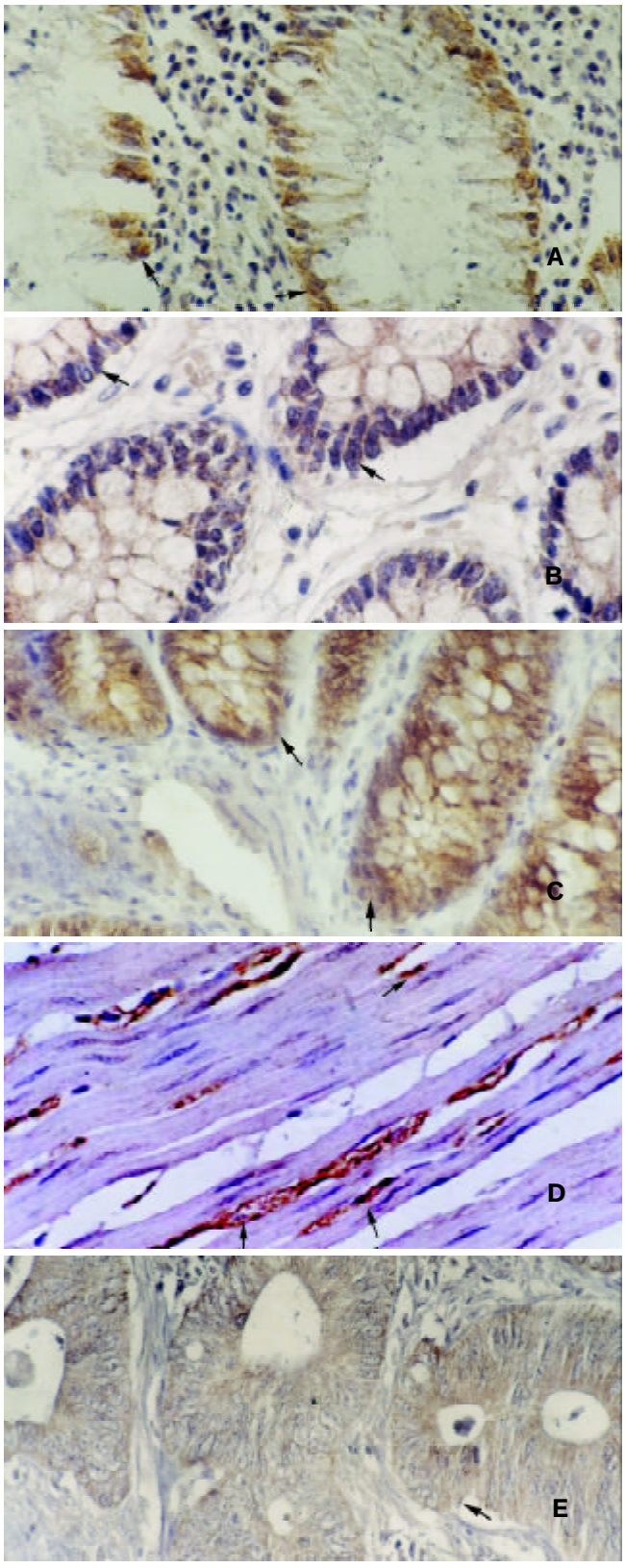

Figure 2 Immunohistochemical staining of ERβ in CRC and normal colonic mucosa. A: shows the ERβ positive epithelium.×200. B: shows the ERβ positive crypt cell. ×200. C: shows the nuclear and cytoplasmic staining in rectal tubular adenocarcinoma.×200. D: shows the ERβ positive smooth muscle cell and stromal cell. ×400. E shows difuse cytoplasmic staining in CRC. ×400.

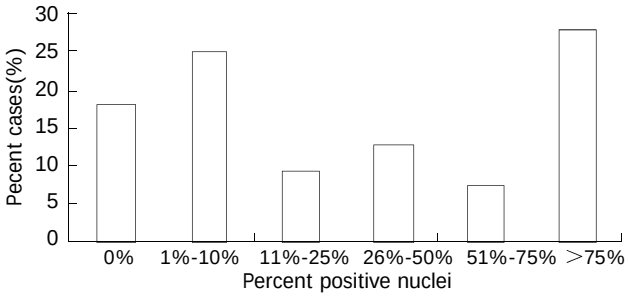

Figure 3 Extent of nuclear ER-β immunoreactivity in 40 cases of CRC.

DISCUSSION

Several epidemiologic studies have shown that colon cancer might be influenced by steroid hormones[1-3], and estrogen use might be associated with a low risk of colon cancer[21-27]. Some experimental results indicated that estrogen had a trophic effect on colon cancer[1,11-12]. However, the effect of estrogen on colon cancer is controversial according to some reports. For example, Qiu *et al*[28] reported that estradiol could induce apoptosis in colo205, a colon cancer cell line expressing only ERβ. The question is whether ER expresses in normal colon mucosa or CRC. Earlier studies using biochemical ER-binding assay concluded that there was no ER in human colon. A more recent study concluded that ER was present in normal human colon and CRC tissues. Several immunohistochemical studies from China or Japan have shown that ER was present in normal human colon and CRC tissues[12-18]. Similarly, our immunohistochemical study using ER antibodies (clone number 1D5), which are specific for ERα, showed that ERα was present in normal colon mucosa and CRC. However, the expression rate of ERα was about 20-40%, which was lower than that of ERβ.

In 1997, the second type of ER (ERβ) was cloned, and recent studies indicated ERβ was distributed in human tissues[5]. Several basic studies have shown that different distribution and regulatory mechanism of these two types of ER played different roles[29,30]. ERβ expression in normal colonic epithelium, especially at the bottom portion of colonic crypts, suggests that estrogens may play an important role in the growth and regeneration of normal colonic mucosa. ERβ expression in a large number of CRCs indicates that estrogens may exert effects on these cancers, which may have significant implications for the treatment and prevention of CRC.

According to some published reports, ERα was also present in gastric or colon cancer[11-18]. Our results support this conclusion. However, the positive rate of ERα in CRC was less than that of ERβ in CRC. Some research results indicated that ERα and ERβ could interact with the fos/jun transcription factor complex on AP1 sites to stimulate gene expression. However, they had opposite effects in the presence of estradiol. In the presence of ERα, estradiol functioned as an agonist in the AP1 pathway. In contrast, in the presence of ERβ, tamoxifen and raloxifene behaved as fully competent agonists in the AP1 pathway, while estradiol acted as an antagonist, inhibiting the activity of both tamoxifen and raloxifene[29,30]. It is the presence of two ERs that explains the conflicting experimental results. We deduced that estradiol might have trophic effects via combining with ERα. However, estradiol could inhibit tumour growth by combining with ERβ.

In this study, we found no significant correlation between ERβ expression and clinicopathologic features, including

Duke's types, lymph node metastasis and differentiation. Because of the relatively small sample size in this study, a study using a larger sampled study is necessary to further investigate the relationship between ERβ expression and clinicopathologic characteristics and survival of colorectal cancer patients.

Our RT-PCR results showed that ERα and ERβ mRNA were both expressed in CRC, semiquantitative RT-PCR revealed there was no statistical significance in ERβ mRNA level between CRC tissue and paired normal colon tissue. Our immunohistochemical results showed that some sections were only cytoplasmically stained. Foley *et al*[33] reported that Western blot analysis revealed very low levels of ERα protein in tumor and normal colon tissue. However, malignant colon tissue showed a selective loss of ERβ protein expression when compared to normal colon tissue in the same patient. A post-transcriptional mechanism may account for the decrease of ERβ protein expression in CRC tissue. Another reason is the different expressions of ERβ isoforms in CRC. There are at least 5 different ERβ isoforms, which show different amino acid sequences at the COOH terminus and are differently expressed in tumor cell lines[31-35]. Campbell-Thompson *et al*[35] and Witte *et al*[36] showed that ERβ was the predominant ER subtype between human colon and that the decreased levels of ERβ1 and ERβ2 mRNA were associated with colonic tumorigenesis in females. Their data suggest that there is a change in the relative expression of ERβ isoforms. Therefore, It is possible that the cytoplasmic immunoreactivity in CRC tissue is caused by one of the overexpressed ERβ subtypes. Further study should determine not only whether there are different ERβ isoform expressions between normal colon and CRC, but also whether different isoforms are associated with different responses to estrogens and antiestrogens.

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