Preliminary Study on the Expression of Estrogen Receptor Beta (ERβ) in Colorectal Carcinoma

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Abstract

Colorectal carcinoma (CRC) has been an important public health problem. It stands at the top of oncologic pathology and it is the third most common cancer diagnosed in men and women in the world. Previous researchers found that the risk of developing CRC is slightly lower in women than in men. This suggested that the hormone estrogen plays a role in the development of CRC. Estrogen receptor beta (ERβ) is the predominant estrogen receptor (ER) in human colon. Therefore, it is reasonable to study ERβ status and the physiological significance of these ERβs in colorectal cancer. The objective of this study was to determine the expression of ERβ in CRC patients and its relationship with the age and gender. ERβ expression in CRC was investigated by immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) tissue sections from 10 CRCs patients. The association between ERβ expression with the age and gender was evaluated by using Pearson’s Chi Square Test. The results of the immunohistochemistry (IHC) demonstrated that out of these 10 cases studied, most of them (70%) were ERβ positive whereas three out of the cases (30%) were ERβ negative. About 57.14% (n=4) and 42.86% (n=3) were scored as 2+ and 3+ respectively. No significant association (P>0.05) was noted between ERβ expression with the patients’ age (P=0.778) and gender (P=0.490). In conclusion, many CRCs cases were positive for ERβ. These results highlighted the presence of ERβ in CRC tissue samples and it is suggested to introduce them as a potential application for early diagnosis, staging, prognosis and treatment of CRC. Studies with a larger number of sample sizes using standardized tests are needed to understand the exact biological role of ERβ in CRC.

Keywords: estrogen receptor beta, colorectal carcinoma, immunohistochemistry.

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Introduction

In both men and women, colorectal carcinoma (CRC) is one of the three most common type of cancers diagnosed after lung and breast cancer. The probability of colorectal carcinoma is found to be relatively higher in men than in women. Generally, the risk is approximately one in 21 (4.7%) for males whereas one in 23 (4.4%) for females throughout their lifetime. The study of colorectal carcinoma is one of the main concerns for researchers around the world due to the high incidence of the disease in today's world. Currently, researchers tend to have the opportunities in investigating colon imaging and conducting biopsy sampling to be used for histopathological and genetic studies.

The process of intestinal carcinogenesis is the final outcome of a multistep dynamic process arising from genetic modifications or mutations that are caused by the environmental influences, particularly in dietary patterns and host specific influencing factors such as hormones and cytokines, including sex steroid hormones such as estrogen. Estrogens have been shown to be involved in multiple pathophysiological disorders, including cancers. Previously, estrogens are known to be involved in carcinogenesis in reproductive organs such as ovaries, and in non-reproductive tissues involving various types of cancers like the gastrointestinal cancer and breast cancer. Estrogens control the metabolism of bile acids. It also decreases the level of insulin-like growth factor in the bloodstream and has a direct effect on the colon mucosa. Because estrogens have many functions in our body system, they need mediators to help them function properly. It is where the estrogen receptors (ERs) functioned by mediating the effects of the estrogens. There are two forms of estrogens that have been identified where both have identical structures, which are estrogen receptor alpha (ERα) and the estrogen receptor beta (ERβ). Both are nuclear receptors, which will bind to the ligand and then digest and translocate it into the nucleus. They control the transcription of target genes through the binding of estrogen-response (ERE) elements towards the DNA molecules. These receptors can further interact with other transcription factor complexes such as activating protein-1 (AP1) and stimulating-protein 1 (Sp1) in the transcription process called transcription factor crosstalk.

Within human colonic epithelium, the predominant ER is the estrogen receptor beta (ERβ). The effects of ERs are mediated by binding to estrogen response elements (ERE) in gene promoters directly but also through activator protein (AP-1) and SP-1 sites indirectly. Previous studies have shown that ERβ expression is reduced during colon tumorigenesis as compared to normal tissue. Furthermore, the ERβ level appears to be inversely associated with the Dukes score and the tumour differentiation grade. Previous study indicated that hormone replacement therapy could minimize the incidence of CRC, and beta-receptor estrogen (ERβ) has been implicated in this defence. Caiazza et al., investigated on the effects of ERβ on intestinal microbiota, have reported in their research that, the intestinal microbiota is altered in both colitis and CRC relative compared to normal intestines. Nevertheless, it remained to be studied whether the intestinal ERβ affected the intestinal microbiota. Their work has shown that colitis-induced CRC decreases intestinal microbiota diversity and ERβ loss enhances this process in the mouse model. The results may lead to new therapeutic or preventive approaches in order to have a desirable microbiome in the development of colorectal cancer treatments. It is therefore believed that estrogen-mediated signalling plays a protective role in CRC. Hence, the purpose of this study was to determine the expression of estrogen receptor beta (ERβ) in CRC patients and its relationship with their age and gender. Further understanding on the roles and biological pathways of ERβ may support the prevention of colorectal cancer in the future and provide potential therapeutic options for patients with ERβ-positive tumours.

Methodology

Tissue samples

Ethics approval was obtained from the Medical Research and Ethics Committee (MREC), Ministry of Health (MOH) Malaysia [NMR-17-2571-38679 (IR)]. A total of 10 colorectal carcinoma specimens were obtained from patients who underwent surgical treatment in the year of 2017 at the Department of Surgery at Hospital Sultanah Nur Zahirah, Terengganu, Malaysia. The formalin fixed paraffin embedded (FFPE) tissue blocks of these cases were collected from the archives of the Department of pathology. Patients that were clinically suspected with hereditary nonpolyposis colorectal cancer associated with inflammatory bowel disease were excluded from this study. Informed consent was obtained from all the patients before performing this study in order to use the tissue samples for research purpose.

Trimming and Sectioning

In prior of preparing tissue sections, the FFPE blocks were chilled on ice. Upon embedding the tissues, they were cut into sections of desired thickness using a microtome (Leica, Germany). The tissues were trimmed at eight micrometer (μm) and sectioned at three micrometer (3μm). Once sections were obtained, they were floated on the warm water bath set at 42°C that helped straightened any wrinkles of tissue sections formed. Then they were affixed on a poly-prep, poly-L-lysine coated glass slides (Sigma-Aldrich, USA).

Immunohistochemical staining

Immunohistochemical staining of estrogen receptor beta (ERβ) antibody was performed on the tissue sections by using Dako REAL™ Envision™ Detection System, Peroxidase/DAB+, Rabbit/ Mouse (Dako, Denmark). For IHC, the paraffin sections were incubated in hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases after deparaffinization in xylene, rehydration
in ethanol and pre-treated by performing heat induced epitope retrieval (HIER).

After the tissue sections were deparaffinized in xylene I and II for five minutes each, they were rehydrated in a graded series of ethanol (100%, 95%, 80%, 70% and 50% alcohol for two minutes each). The slides were immersed in Envision FLEX Target Retrieval Solution High pH (Dako, Denmark) and placed into microwave set at high power for 20 minutes. The slides were then left to cool down under running tap water and incubated in Dako Wash Buffer (Dako, Denmark).

Then, the sections were proceeded to staining. Breast carcinoma tissue was used as a positive and negative control as breast tissue had been shown to express positive ERβ staining. For negative controls, the tissue sections were incubated with wash buffer solution instead of primary antibody. The tissue sections were incubated with 200 microliter (μl) of Hydrogen Peroxide Block (Dako REAL™ Peroxidase-Blocking Solution; Dako, Denmark) for 15 minutes and rinsed with wash buffer for three times and incubated for three minutes with the buffer. Tissue sections were then incubated with 200μl of primary antibodies anti-ERβ (Abcam, United Kingdom), in the dilution ratio of 1:200 for overnight (16-18 hours) at 4°C. It was then rinsed and incubated in wash buffer for three minutes. The tissues were then incubated with two drops of Dako REAL™ HRP Conjugate (Dako, Denmark) each for 30 minutes, then washed and incubated in wash buffer for three minutes. Freshly prepared of 200μl Dako REAL™ DAB+ Chromogen (Dako, Denmark) were added and tissues were incubated for 10 minutes. They were then rinsed for three times and incubated in wash buffer for three minutes. All of the tissue sections were then counterstained with Leica Surgipath Hematoxylin 560 MX (Leica, USA) for a minute and washed under running tap water before proceeded with dehydration. The slides were mounted with Richard-Allan Scientific Cytoseal™XYL (Thermo Scientific, USA) and covered with cover glasses 20 × 40 mm (Hirschmann®, Germany). The slides were then observed under microscope.

**Evaluation of immunoreactivity**

Immunoreactivity detected in the cytoplasmic and nuclear were evaluated according to the modified Histoscore (H-score) method as previously reported[2]. Relative stain intensity in the colorectal carcinoma was scored as follows: 0=negative, 1=weak, 2=intermediate, or 3=strong. Briefly, a score of zero indicated no staining observed in tumour cells. A score of 1+ indicated weak, incomplete nuclear staining in any percentage of tumour cells or weak, complete nuclear staining in fewer than 10% of tumour cells. A score of 2+ indicated weak, complete nuclear staining in 10% or more of tumour cells or intense, complete nuclear staining in fewer than 30% of tumour cells. A score of 3+ indicated uniform, intense, complete nuclear staining in more than 30% of tumour cells. H-score were subsequently generated by adding the percentage of area occupied by cells scored with 3x3, the percentage of area occupied by the cells scored with 2x2, and the percentage of area occupied by the cells scored with 1x1, giving a possible range of 0-300.

**Statistical analysis**

The data were analyzed using IBM SPSS Statistics Version 22 computer program. The association between estrogen receptor beta (ERβ) and clinicopathological factors was evaluated using Pearson’s Chi Square Test, and a P-value less than 0.05 was considered as statistically significant.

**Results**

**ERβ Expression in Colorectal Carcinoma Patient**

This retrospective study was conducted on colorectal tissue resection specimens collected from 10 patients diagnosed with colorectal carcinoma (CRC). Tissue samples included in this study were from five males (50%) and five females (50%) with a median age of 65 years old (range: 55-75 years) as shown in Table 1. Studying on the distribution of patients’ ages, separately for the two sexes, these were the following results: maximum incidence in men was found to be in the age group of 65-70 years (three patients, 60% of the group of men) whereas in women maximum incidence was between 55 to 60 years (three patients, 60% of women). Most of the ERβ positive cases (four cases) were males. Most of the ERβ positive patients were aged between 55 to 70 years.

Immunohistochemical staining for ERβ was performed in all of the ten cases of CRC and scored where the results were taken based on the intensity and the pattern of ERβ expression. Most of them were ERβ positive (70%), while 30% of them were ERβ negative (Table 2).

**ERβ Immunoreactivity**

ERβ immunoreactivity was detected in the nuclear and cytoplasmic of the colorectal carcinoma cells (Figure 1). Table 3 showed the results of semiquantitative determination of ERβ immunostaining intensity of the specific cases. The status of ERβ immunoreactivity was positive in seven of the 10 colorectal carcinoma cases included in our study. Most of them were ERβ positive (70%). From the positive cases, 30% (n=3) were scored as 3+, 40% (n=4) were scored as 2+ and none (n=0) were scored as 1+ respectively. No visible ERβ immunoreactivity was detected in the negative control sections included in this study.

**Association between ERβ Expression with the age and gender**

Associations between the immunohistochemical ERβ status with the age and gender of the colorectal carcinoma patients were analyzed by using Pearson’s Chi Square Test utilizing IBM SPSS Statistics Version 22 computer program. It was summarized in Table 4. There was no...
significant association between the ERβ status and the patients’ age ($P=0.778$) and gender ($P=0.490$). No statistically significant relationship was detected between the scoring of ERβ and age of patients ($P>0.05$). There was no significant correlation between gender and ERβ positive reaction ($P>0.05$).

**Discussion**

The level of ERβ overexpression in patients with CRC ranges from 10% to 86% as proven in previous studies. Recent studies showed that ERβ is positive in almost 50% of CRC tissues. However, in our study showed about 70% of the colorectal carcinoma tissues were positive immunoreactivity. These data seem contradict as compared to other related studies may be due to small sample size were being studied. A study by Li-Qun Xie et al. showed that about 57.5% of CRC tissues and 20% of normal colorectal tissues were ERβ positive. This showed that ERβ expression is higher in CRC compared to the normal colorectal tissues. Hassan et al. stated that only 56% of CRC cases demonstrated ERβ positive immunostaining, whereas the rest of 44% of the cases were ERβ negative. So, it is not clear whether ERβ has the potential to be developed as the therapeutic target or prognostic marker in CRC patients.

In present study, about 50% of the CRC cases studied were males and another 50% were females. The results obtained were not in accordance with most of the previous studies. Soderlund et al. showed differences in the incidence of colorectal cancer between gender where the female population had a lower risk compared to male population. Some researchers reported that males had a higher incidence while on the other hand, Cresssey et al. had showed that females had a higher incidence of CRC. Variation between different studies may be related to the randomized selection of samples and having a small sample size. It is important to note that the mentioned studies had used various scoring systems or different antibodies, which makes the findings significantly inconsistent.

Looking into the distribution by age consecutively for both sexes, the results showed maximum incidence in men was in the age group of 65 to 70 years (three patients, 60% of the group of men) whereas in women maximum incidence was between 55 to 60 years (three patients, 60% of women). The ages of patients in this study ranged from 55 to 75 years (mean age of 64.7 years old) where all (100%) of the patients were older than 55 years. These results are relatively compatible with the findings by Neklason et al. which stated that the mean age of colorectal carcinoma patients in their study were 58.3 years old and Shabbir et al. who reported the mean age of colorectal adenocarcinoma patients were 56 years.

The association between ERβ expression with the age and gender were determined by using Pearson’s Chi Square test. The P-value which is less than 0.05 was considered as significant. However, in this study, there is no significant association found between ERβ expression with the age and gender. These findings are relatively compatible with several previous studies. The P-value of patient’s age is 0.778 and the P-value of patient’s gender is 0.490, which is statistically insignificant. This indicated that the expression of ERβ in colon carcinoma tissues can occur in all patients of any ages and gender. Although our sample size was small, our results were compatible with some studies with large number of cases. For example, Li-Qun Xie et al. found that there was no association between ERβ expression and clinicopathologic features such as age, gender, lymph node metastasis and tumour grading.

To conclude, present study showed no association between the expression of ERβ with age and gender. It is essential to note that most studies used colorectal carcinoma criteria for scoring the expression of ERβ in CRC; however, because of significant differences in biological origins of these cancers, it needs to be exactly clarified by further investigations whether it is suitable for treatment of CRC or not.

There were two major challenges which mark the future of this field of research. Firstly, it is crucial to further characterize the expression of ERβ in CRC patients by identifying a specific subset of patients with reduced ERβ loss or differential expression of ERβ isoforms in order to assist in stratification and patient selection for therapies targeting estrogen signaling in future. Secondly, the downregulation of ERβ and the subsequent loss of protective signaling in advanced tumours warrant further investigation into the mechanisms involved to address the potential for onco-suppressive signaling to be reactivated therapeutically. A positive feedback mechanism has been described in vitro where stimulation of estrogen resulted in increased expression of the ERβ protein, reinforcing pro-apoptotic signals in colorectal cancer cells.

While estrogen modulation plays a role in the prevention of colorectal cancer, the burden lies on researchers to identify the appropriate population and product to move the field forward. Several investigators have confirmed the presence of ERβ in the normal and colonic epithelium, colon cancer cell lines and human colorectal cancer tissues. The function of ER in both normal and malignant colon, however, remains unknown to date. The present study suggested that ERβ may mediate the protective effects of estrogens against colorectal carcinoma. But due to some limitations such as using a smaller sample size in this study might have drawbacks to verify this. Therefore, we suggested for the need of prospective, large-scale and multicentred studies in order to confirm on the results obtained. In addition, the mechanism behind the prognostic value in CRC remained unclear. Additional studies regarding the role of ERβ in CRC are required. Therefore, further studies on the molecular pathways and the biological functions or role are necessary in order to have a better understanding on the effects of ERβ expressions in colorectal cancer.

**Conclusion**

ERβ was expressed in colorectal carcinoma tissues and this suggested that ERβ plays an important role in colorectal carcinoma. However, it is not clear whether it plays a protective role or a causative agent. In conclusion, present study showed about 70% of the colorectal carcinoma tissues showed ERβ positive...
immunoreactivity. No significant association was detected between the expression of ERβ with the age and gender. Thus, studies with a larger population and research on ERβ isoforms variety are required in order to obtain results with a higher statistical significance. Further investigations are needed to exactly clarify whether it has potential for treatment of CRC and to understand as it might be a novel treatment for the colorectal carcinoma patients detected with positive ERβ expression.

**Conflict of Interest**

The authors declared that there is no conflict of interest.

**Acknowledgements**

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**References**


Table 1. Summary of colorectal tissues samples

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Range of age</th>
<th>Mean age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal Carcinoma (n=10)</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>55-75</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 2. Expression of ERβ in CRCs

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ERβ positive (%)</th>
<th>ERβ negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC</td>
<td>10</td>
<td>7 (70%)</td>
<td>3 (30%)</td>
</tr>
</tbody>
</table>

*The number represented the number of cases.

Figure 1. Immunohistochemical staining of Erβ positive immunoreactivity. (A) Breast carcinoma tissue as positive control at magnification of 400x. (B) Colorectal carcinoma tissue at magnification of 400x.
Table 3. Semiquantitative determination of the immunostaining intensity of ERβ

<table>
<thead>
<tr>
<th>No of cases</th>
<th>Erβ immunoreactivity scorea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
</tr>
</tbody>
</table>

*aEstrogen receptor beta immunostaining score intensities were represented as follow: high ++++, medium ++, low + and negative non-detectable staining ---.

Table 4. Age and gender of patients with CRCs and their association with ERβ expression

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>ERβ positive* (%)</th>
<th>ERβ negative* (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>4</td>
<td>3(75.0)</td>
<td>1(25.0)</td>
<td>0.778</td>
</tr>
<tr>
<td>&gt;60</td>
<td>6</td>
<td>4(66.6)</td>
<td>2(33.3)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td>0.490</td>
</tr>
<tr>
<td>Male</td>
<td>5</td>
<td>4(80.0)</td>
<td>1(20.0)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>3(60.0)</td>
<td>2(40.0)</td>
<td></td>
</tr>
</tbody>
</table>

*The values represented the number of cases. *P*<0.05 was considered as significant.