RESEARCH ARTICLE

Tumor Differentiation is Correlated with Estrogen Receptor Beta (ERβ) Expression but Not with Interleukin-6 (IL-6) Expression in Colorectal Carcinoma

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Received date: Sep 10, 2024; Revised date: Oct 21, 2024; Accepted date: Oct 24, 2024

Abstract

BACKGROUND: Colorectal carcinoma (CRC), the third most common malignant disease worldwide, is associated with estrogen receptor β (ERβ) and interleukin-6 (IL-6). ERβ is known to down-regulate IL-6 in prostate cancer, lung carcinoma, and CRC cell lines; however, its effect on human with CRC remains unclear. Therefore, this study was conducted to investigate the association between ERβ and IL-6 expressions with the clinicopathological features of CRC.
METHODS: This was an analytic observational study using 40 paraffin blocks of CRC patients. ERβ and IL-6 expression was measured by immunohistochemistry (IHC) staining. The percentage of immunoreactive tumor cell per 1000 cells was manually recorded and tumor differentiation as well as tumor infiltration were determined. Tumor differentiation was graded according to the World Health Organization (WHO) 2010 criteria, while tumor infiltration was defined based on the American Joint Committee on Cancer (AJCC) 8th edition.
RESULTS: Fifty percent of samples were well-differentiated CRC, and 57.5% samples were T3 infiltration tumors. IHC staining showed 35.5% of samples were positive for ERβ expression, while 70.86% were positive for IL-6 expression. There were negative correlation of ERβ expression with tumor differentiation (p=0.018; r=-0.371), but no correlation with tumor infiltration (p=0.836) were found. There was no correlation between ERβ expression with IL-6 expression (p=0.154).
CONCLUSION: There is statistically significant correlation between tumor differentiation and ERβ expression, wherein improved tumor differentiation is linked to higher levels of positive ERβ expression. However, there is no discernible relationship between IL-6 and tumor differentiation. These findings suggest that while IL-6 was involved in the growth of the tumor, ERβ expression might have an impact on tumor differentiation.
KEYWORDS: colorectal carcinoma, estrogen receptor beta, interleukin-6, cell differentiation

Indones Biomed J. 2024; 16(5): 481-6

Introduction

The number of colorectal carcinoma (CRC) cases has been rising, ranking third globally in 2018, with 1.8 million new cases per year (10.2%).(1) CRC ranks as the third most prevalent type of cancer CRC is also the second leading cause of cancer-related deaths across the globe.(2) The incidence of CRC is 30% higher in men than in women, with men also showing an earlier onset and a higher prevalence of

adenomas.(3) Some studies also indicates that CRC is more prevalent in men than women. This fact leads to a suspicion regarding estrogen's effect on the development of CRC. Studies suggest an inverse relationship between estrogen receptor beta (ERβ/ESR2) and the incidence of colorectal polyps and tumor stages, potentially offering a protective effect. About two-thirds of sporadic CRC cases arise from adenomas with polypoid features.(1,2) Both lab and human trials have shown that substances like phytoestrogens and drugs targeting ERβ can trigger these receptors, promoting cell death and reducing CRC incidence.(4,5) This underscores the protective role of estrogen against CRC.

Pro-inflammatory cytokines, such as interleukin (IL)-6, have also been linked to increased CRC risk and tumor progression.(6,7) Inflammation raises free radical levels, leading to DNA damage and potential neoplastic transformation.(8,9) However, the precise mechanism of CRC development involving the estrogen receptor and IL-6 pathway remains unclear. Early detection methods, including identifying prognostic biomarkers and developing new therapies, are crucial for CRC. Further research on ERβ and IL-6 expressions in CRC development is needed to determine which pathway is more dominant and whether there is a connection between ERβ and inflammation via IL-6. If such association exists, ERβ and IL-6 might become the next therapeutic targets and prognostic biomarkers for CRC patients.

Various therapies including chemotherapy, radiotherapy, or surgery, faced some difficulties in combating cancer cells.(10) Several studies suggest that drugs like estrogen, which is used in postmenopausal hormone replacement therapy, and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, may help preventing the development of CRC. Cohort studies indicate that regular, long-term use of aspirin and other NSAIDs can reduce CRC risk. However, the American Cancer Society does not recommend NSAIDs for cancer prevention due to some risks such as gastrointestinal bleeding. Similarly, postmenopausal hormone therapy is not advised for CRC prevention, as it may increase breast cancer and cardiovascular disease risks. Currently, the American Cancer Society advises against using drugs or supplements for CRC prevention because the correct dosage, effectiveness, and potential toxicity are unclear.(11) Although estrogen may influence CRC proliferation and suppression, the exact pathways remain unknown, prompting further research into its association with the inflammatory process in CRC development.

ERβ is the dominant estrogen receptor in the colon mucosa.(12) Research using CRC cell lines with ERβ shows that it can down-regulate IL-6 through NF-κβ in SW480 cells. Estrogen has been shown to reduce IL-6 expression in various cell line cultures by inhibiting protein binding in NF-κβ.(13) In multiple myeloma, estrogen inhibits IL-6-induced cell proliferation.(14) Estrogens may have anti-tumor effects by selectively activating ERβ-mediated pro-apoptotic signals, inhibiting inflammatory signals, and modulating the tumor microenvironment.(15,16) Even though, understanding the interaction between ERβ and IL-6 in CRC is important to consider the usage of ERβ agonist (Silymarin) and IL-6 inhibitors in CRC as targeted therapy, unfortunately limited studies are available to explain the mechanism. Therefore, this study was conducted to investigate the differences and correlation of ERβ and IL-6 expressions in the development of CRC in term of tumor differentiation and tumor infiltration.

Methods

Study Design and Samples Collection

This was an analytic observational study with cross-sectional design using 40 paraffin blocks of CRC patients that were obtained from the Anatomical Pathology Department of Dr. Soetomo General Hospital, Surabaya. Paraffin blocks of resected CRC patients that were in good condition were included as samples in this study. However, paraffin blocks of patients who received neoadjuvant chemotherapy and patients diagnosed with mucinous adenocarcinoma colon were excluded. Samples that met the inclusion and exclusion criteria further randomized and analyzed for the expression of ERβ and IL-6.

The protocol of this study was reviewed and approved by the Ethical Review Committees of Dr. Soetomo General Hospital (Ref No. 0461/LOE/301.4/V/2021). This study has also been registered in ClinicalTrial.gov ID (No. NCT05202548). Since in this study samples were taken from the remaining material of a pathology laboratory specimen, hence no explicit formal informed consent was obtained from patients according to hospital protocol.

Immunohistochemistry (IHC) Analysis for ERβ and IL-6 Measurement

Each specimen was cut into 5 µm-thick parallel sections and stained with hematoxylin and eosin. After deparaffinization and rehydration, the sections were briefly microwaved for antigen retrieval using citrate buffer, pH 6·0, at 750 W for 10 minutes, and at 350 W for 15 minutes. After being cooled down for 20 minutes at room temperature, the sections were

incubated with hydrogen peroxide for 10-15 minutes and then washed two times with buffer. Then, the sections were incubated separately with primary antibodies according to the manufacturer's protocol. The Anti ERβ Polyclonal Antibody (Cat. No. bs-0255R; Bioss Antibodies, Woburn, MA, USA) and Anti-IL-6 Monolyclonal Antibody (Cat. No. E-AB-30095; Elabscience, Houston, TX, USA) were used. After the incubation with primary antibodies, a 10 minute incubation with Biotinylated Goat Anti-polyvalent (Cat. No. E-AB-1097; Elabscience) and a 10 minutes incubation with Streptavidin – Horseradish Peroxide (HRP) (Cat. No. 434323; Thermo Fisher Scientific, Waltham, MA, USA) were performed. Lastly, a mixture of 40 µL DAB Plus Chromogen and 2 mL of DAB Plus substrate was applied into tissue for 15 minutes. The expressions of ERβ and IL-6 was manually counted in ten fields of view, using 400x times magnification by two pathologists.

Determining Tumor Differentiation and Tumor Infiltration

The percentage of immunoreactive tumor cells per 1000 cells was manually recorded. Tumor differentiation was graded according to the World Health Organization (WHO) 2010 criteria, and tumor infiltration was defined based on the American Joint Committee on Cancer (AJCC) 8th edition.(17) For the statistical analysis purpose, in this study, the tumor primer invasion was grouped into T1-T2 group, which were the tumor invasion into submucosa (T1) and into muscularis propria (T2), as well as T3-T4 group, which were the tumor invasion into subserous (T3) and into peritoneum (T4).

Results

Clinical Characteristic of Samples

From the year of 2019, among 64 patients undergoing colon resection in Dr. Soetomo General Hospital, 40 samples fulfilled the inclusion and exclusion criteria. There was no difference in the number of male and female patients (Table 1). The highest incidence of colorectal carcinoma was found in patients in their 61-70 years old (13%), with a mean of 54.13±13.96. The youngest patient was 27 years old, while the oldest was 78 years old.

Based on the microscopic tumor differentiation analysis, 50% of the samples in this study were well-differentiated, and the rest were moderate- and poor-differentiated (Table 1). The most tumor size was >5 cm (60%), and in 57% of patients, the tumor had infiltrated into the peri colorectal tissues (T3). Interestingly, 45% of the subjects have not had tumor metastases to the lymph nodes. The evaluation results of ERβ IHC staining showed an average of 35.5% of the colonic mucosal tissue were carcinoma positive for ERβ and 70.86% were positive for IL-6 (Table 1, Figure 1, Figure 2).

Tumor Differentiation was Correlated with ERβ Expression

There was a significant difference between well-, moderate-, and poor-differentiation based on the ERβ expression (p=0.018), with better tumor differentiation (well-differentiated) was associated with higher levels of positive ERβ expression (37.96±9.43). In contrast, there was no discernible difference between the well-, moderate-, and poor-differentiation based in the IL-6 expression (p=0.486) (Table 2).

Using the Spearman Correlation Test, the correlations between ERβ expression and tumor differentiation was also found to be significant (p=0.018). However, there were no

Table 1. The clinical characteristics pathological features of samples (n=40).

Characteristic	Value
Gender, n (%)	
Male	20 (50)
Female	20 (50)
Age (year), n (%)	
21-30	1 (2.5)
31-40	4 (10)
41-50	8 (20)
51-60	10 (25)
61-70	13 (32.5)
71-80	4 (10)
Microscopic Differentiation, n (%)	
Well-differentiated	20 (50)
Moderately differentiated	17 (42.5)
Poorly differentiated	3 (7.5)
Tumor Size (cm), n (%)	
<3	4 (10)
3-5	12 (30)
>5	24 (60)
Tumor Infiltration, n (%)	
T1 (limited to the submucosa)	1 (2.5)
T2 (through muscularis propria)	9 (22.5)
T3 (through muscularis propria into pericolorectal tissues)	23 (57.5)
T4 (through visceral peritoneum or other organs)	7 (17.5)
Metastases to Regional Lymph Node (N), n (%)	
N0 (no regional lymph node metastases)	18 (45)
N1 (metastases in 1-3 regional lymph nodes)	14 (35)
N2 (metastases in >3 regional lymph nodes)	8 (20)
Expression per 1000 cells, mean±SD	
ERβ	35.5±8.5
IL-6	70.86±14.1

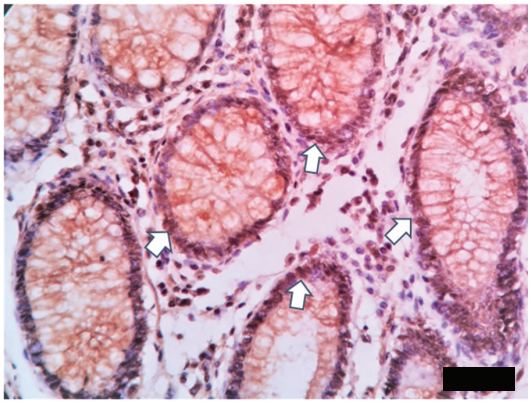

Figure 1. Example of ERβ expression in mucous of CRC subject. White arrows referred to positive ERβ expression in the nucleus. Black bar: 10 µm.

correlations between ERβ expression and tumor infiltration (p=0.836), as well as between ERβ expression and IL-6 expression (p=0.154) (Table 3). This results suggested that the mechanism of tumor differentiation might indeed involve the regulation of ERβ expression, but not IL-6.

Discussion

In this study the highest incidence of CRC cases was found in patients aged 61-70 years old. The patient characteristics obtained from this study did not differ much from the characteristics of CRC patients at Sanglah Hospital, Bali, where 59.5% of patients were male, and the most age group was 50-60 years old.(18) However, the results of this study showed that around 32% patients were under 50 years old, with the youngest age was 27 years old. This was consistent with earlier research showing an increase in early-onset colorectal cancer (EOCRC) worldwide. Moreover, there are 16% to 35% of EOCRC instances in individuals with hereditary cancer diseases.(19,20)

In the current study, it was found that 75% of patients are in the tumor infiltration stage that had penetrated the serosa, or T3-T4 stage according to the Tumour, Node, Metastasis (TNM) staging from the AJCC, and even 55% had metastases to mesenteric lymph nodes either N1 (1-3 regional lymph nodes) or N2 (>4 regional lymph nodes), indicating that the patient had entered at least stage IIIA17. Furthermore, 60% of patients had tumors with a maximum diameter of >5 cm. This demonstrates a lack of awareness for carcinoma screening, and patients seek for clinical help only after the obstruction symptoms developed. It has been well known that the primary key to the successful treatment of colorectal cancer is the early detection of malignancy, since no adjuvant therapy is needed in stage 1 patients. Even for the patients in stage 2, adjuvant therapy is only needed in high-risk patients, including <12 lymph nodes resected, poorly differentiated tumors, vascular or lymphatic or perineural invasion, tumors with occlusion or perforation, and those who are in the T4 infiltration stage. (11) Interestingly, 45% of patients did not develop lymph nodes metastases.

This study highlights the significance of ERβ expression in CRC patients, particularly in Indonesia. The f indings indicate that ERβ expression is inversely correlated with tumor differentiation, suggesting that increased ERβ expression is associated with better tumor differentiation. This is consistent with previous studies that have shown similar results in low-grade CRC, glial tumors, and prostate adenocarcinoma.(5,21) Estrogen receptor had a significant inverse correlation with tumor differentiation, which suggests that increased ERβ expression leads to better tumor differentiation. In accordance with our results, another study also showed that ERβ expression was considerably higher in low-grade CRC, which supports our findings.(12) Furthermore, there is an inverse relationship between ERβ and tumor grade in glial tumors (22) and prostate adenocarcinoma (21). These findings suggest that ERβ expression could be used to assess the prognosis for CRC. Consequently, in order to validate the relationship between ERβ expression and CRC patients' survival rate and disease progression, further cohort research is required.

IL-6 is a pro-inflammatory cytokine that plays a significant role in the tumor microenvironment.(23) High IL-6 levels promote tumor growth, invasion, and metastasis.

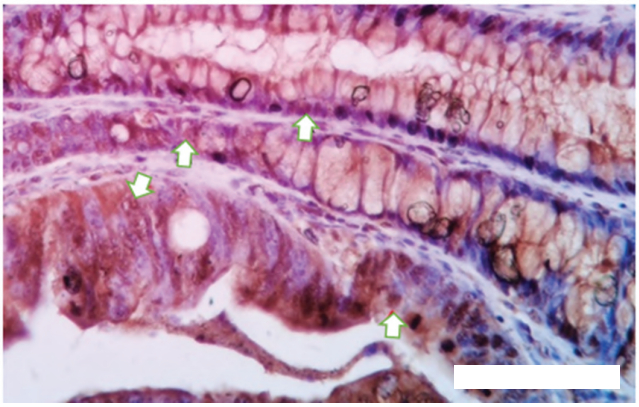

Figure 2. Example of IL-6 expression in mucous of CRC subject. White arrows referred to positive IL-6 expression in the nucleus. White bar = 10 µm.

Table 2. Comparison of ERβ and IL-6 expression within tumor differentiation.

Differentiation	ERβ Expression (mean±SD)	*p*- value	IL-6 Expression (mean±SD)	*p*- value
Well	37.96±9.43		75.22±13.07	
Moderate	32.51±6.8	0.018*	64.91±13.49	0.486
Bad	32.47±0.93		88.20±7.99	

Tested with Independent sample t-test. *$p \leq 0.05$ is considered to be significant.

It activates various signalling pathways, such as the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, which enhances cell proliferation and survival.(24) IL-6 also contributes to an immunosuppressive tumor microenvironment, that influence the differentiation of immune cells. In this study, it was also found that IL-6 expression was high in poorly differentiated CRC. This result is consistent with the report from previous study stating that IL-6 expression was higher in poorly differentiated CRC than well-differentiated CRC.(7) Elevated IL-6 levels in CRC patients are associated with important implications for tumor progression. The IL-6 signaling pathway plays a critical role in promoting several malignant processes in tumor cells, including cell cycle progression, proliferation, anti-apoptotic mechanisms, metastasis, and angiogenesis. (25) However, further cohort research with larger samples might be necessary to be conducted to understand how IL-6 is correlated with these mechanisms.

Conclusion

The results of this study showed that there is statistically significant correlation between tumor differentiation and ERβ expression, wherein improved tumor differentiation is linked to the higher levels of positive ERβ expression. Although IL-6 expression was found to be high in poorly differentiated CRC, but there was no discernible relationship between IL-6 and tumor differentiation. These findings suggest that while IL-6 was involved in the growth of the tumor, ERβ expression might have an impact on tumor differentiation.

Acknowledgments

Special thank to Departement of Pathology Anatomi Dr. Soetomo General Hospital Indonesia for providing the research samples. Thank you to dr. Diah, dr. Ety, dr. Willy for their kind suggestion during the study discussion.

Authors Contribution

IT, IKS, VSB, and BDN were involved in the conception and design of the study. IT, FE, YJH, and BDN analyzed and interpreted the data. BDN performed the statistical analysis of the daya. IT and BDN drafted the original article, as well as revised the article for important intellectual content. IT is responsible for obtaining the funding. VSB and IKS supervised the study and gave final approval.

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Table 3. The associations of ERβ, IL-6 and the clinicopathological form of CRC.

Expression	IL-6	Differentiation			Infiltration
		Well-poor	Moderate-poor	Moderate-well	
ERβ	r = 0.230		r = -0.371		r = 1.009
	p = 0.154		*p* = 0.018*		*p* = 0.836

Tested with Spearman Correlation test. *$p \leq 0.05$ is considered to be significant.

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