THE DEGREE OF COLORECTAL CANCER DIFFERENTIATION AND INFILTRATION WITH TISSUE INTERLEUKIN 6 EXPRESSION

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ABSTRACT

Introduction: Colorectal cancer (CRC) is one of the most common malignancies reported in Indonesia, and it takes the fourth position as the most prevalent cancer just after lung carcinoma. The diagnosis of nm is established through advanced examination such as colonoscopy, although other supporting examinations, consisting of occult blood test and histopathology examination. Pathogenesis of CRC involves the pro-carcinogenic factors (IL-6, IL-1, and TNFα) accompanied by a genetic mutation that predominates the anti-carcinogenic factors; this pathological process underlies the occurrence of CRC.

Purpose: This study aimed to analyze the correlation between the degree of colorectal adenocarcinoma differentiation and infiltration with tissue IL-6 expression.

Method: This study was conducted using an observational analytic method with a retrospective study design. The degree of colorectal adenocarcinoma differentiation and infiltration were identified through histopathological examination; the absorption rate of pigment-containing anti-IL-6 measured the degree of tissue IL-6 expression. The correlation test was performed by using *the Spearman Rank test* on SPSS version 25.

Results: There was a significant correlation between the degree of adenocarcinoma differentiation with tissue IL-6 expression ($p = 0.039$; < 0.05) but not with the degree of tumor infiltration ($p = 0.129$; > 0.05). Statistical tests also showed a negative, significant correlation between sex and IL-6 expression, indicating a male predominance of IL-6 expression in colorectal adenocarcinoma cases; this finding is supported by a T-test that revealed a significant difference in interleukin-6 expression between male and female sex.

Conclusion: Tissue IL-6 expression is correlated with the degree of colorectal adenocarcinoma differentiation but not with the degree of infiltration.

Keyword: Adenocarcinoma, Differentiation, IL-6, Colorectal Carcinoma

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the cancer that ranks 4th in the incidence of cancer in Indonesia. There is no definite causative factor for colorectal cancer. Worldwide cases of colorectal carcinoma are in 4th place out of 100.000 people, with lung cancer occupying the 3rd rank. In 2020, based on data collected by WHO, colorectal carcinoma cases reached 34,189 cases, with a percentage of 8,6% of all cancer cases.[1]

Colorectal carcinoma in Indonesia mostly affects people with an average age of 40 years, compared to developed countries with an average age of 50 years.[2] The most common tests to diagnose colorectal carcinoma are colonoscopy and occult blood tests. Both of these examinations still have deficiencies; colonoscopy examination is invasive, and occult blood tests have low sensitivity, amounting to 50% with the guaiac-fecal occult blood test (G-FOBT) examination and 75% with the fecal immunochemical test (FIT) examination.[3,4]

Cancer tissue has several characteristics, such as infiltration ability and a relatively short differentiation period. This process is influenced by various factors such as hormones (estrogen), growth factors, and pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα.[5–7] Interleukin 6 (IL-6) is a group of pro-inflammatory cytokines first discovered in 1986. Previous studies suggest that there is an effect of upregulation of an inflammatory cytokine. Cytokine expression of IL-6 is one of the influences of growth factors and tumor invasion.[8–11]

IL-6 can act as a tumor promoter by activating downstream oncogenic transcription factors in epithelial cells, which is Nuclear Factors kappa beta (NF-κB) and signal transducer and activator of transcription (STAT) 3 that are part of STAT. Extracellular IL-6 membrane-bound IL-6 (mIL-6R) combines to form a gp130-binding complex. After activation of gp130, STAT 3 will be phosphorylated tyrosine, homo- or heterodimer, and will move to the nucleus, which contains gene transcription. STAT 3 here will activate many important cells in regulating growth, differentiation, and cell cycle.[5–7]

Ueda, 1994 stated a relationship exists between the expression and intensity of IL-6 in tumor cells. Patients with higher serum IL-6 in patients with CRC compared with normal people.[12] Kinoshita, 1999 also showed an association with the degree of differentiation and tumor infiltration associated with high IL-6 in cancer tissue.[13]

The study conducted by Chung et al. in 2003 also stated that IL-6 levels were increased in patients with colorectal carcinoma, associated with CRC status; the results of the above studies indicate that IL-6 can be used as a marker to monitor cancer stage.[14] Evidence that the expression level and intensity of IL-6 in colorectal carcinoma can be associated with the degree of differentiation and infiltration of colorectal carcinoma.

Colorectal carcinoma is one of the most difficult cancers to detect. Currently, no research in Indonesia studies the level of expression and intensity of IL-6 on the degree of differentiation and infiltration of CRC. This study aims to determine the relationship between IL-6 expression and the degree of differentiation and infiltration of CRC in patients at Dr. Soetomo.

METHOD

This research is an observational analytic study, intending to analyze the correlation between the degree of infiltration and differentiation of colorectal cancer by expression of interleukin – 6. The population in this study was paraffin samples (blocks) from colorectal adenocarcinoma patients available at the Anatomical Pathology Unit of the Laboratory Installation of RSUD Dr. Soetomo, Surabaya in 2019; Meanwhile, the samples in this study included colorectal adenocarcinoma tissue preparations in the form of paraffin

blocks and met the inclusion criteria set by the researchers. The samples were randomly selected and then subjected to specific immunohistochemical staining for IL-6. The inclusion criteria of the study population were paraffin blocks from colorectal cancer, which were resected and legible/undamaged.

This research was conducted at the Pathological Anatomy Unit of the Central Laboratory Installation of RSUD Dr. Soetomo Surabaya under the licensing and ethical supervision of the Health Research Ethics Committee, RSUD Dr. Soetomo Surabaya after being declared ethically fit based on the exemption number Np. 0461/LOE/301.4.2/V/2021, which was made on 7 May 2021.

The first procedure is the collection of data to be examined; the information collected includes the degree of differentiation and tumor infiltration from the software. Collection of paraffin blocks and slides of KKR cancer tissue smeared with H&E staining were then examined for their physical condition and selected based on predetermined inclusion criteria up to 40 samples. The slides are then re-evaluated to determine whether they can be read again; if there are slides that are damaged/unreadable, then the paraffin block is cut again.

The paraffin block from the selected sample was then smeared/stained with an immunohistochemical solution using IL-6 (obtained from Bioss USA, bs-4539R). IL-6 expression is said to be positive if the cell nucleus is brown. The immunoreactive cells were then counted under a light microscope per 1000 cancer cells, and the percentage was calculated.

The process of immunohistochemical examination of available preparations consists of the following steps: tissue/sample preparation, tissue deparaffinization and rehydration, antigen retrieval, and immunostaining. In the preparation stage, the tissue was cut four μm thick, then placed on a glass slide that had been cleaned, then the preparation was heated in the microwave for 45 minutes at 60°C. The deparaffinization step was carried out by washing the sample with xylol two times, each for 3 minutes at room temperature, followed by washing with 1:1 absolute alcohol for 3 minutes and at room temperature. The slides were washed twice for 3 minutes using 95%, 75%, and 50% alcohol at room temperature, respectively.

Antigen retrieval functions allow antibodies from reagents to interact with specific antigens/proteins expressed by tissues. Antigen retrieval was carried out by boiling the slides in 0.01 M sodium citrate buffer (pH 6) at 100°C for 15 to 20 minutes; after boiling, the slides were removed and then immersed in PBS for 20 minutes, at room temperature, followed by rinsing the slides. twice with TBST for 5 minutes at room temperature.

The final stage is immunostaining; this stage is carried out by immersing the slide in 3% hydrogen peroxide solution for 30 minutes, then immersing the sample in 5% serum or BSA for 2 hours at room temperature. The buffer is removed from the slide. The slides were then incubated with the primary antibody for one night at 4°C and washed twice for 5 minutes each using TBST at room temperature. The slide was then soaked with diacetyl benzene chromogen for 15 minutes at room temperature and again washed with distilled water for 1 minute at room temperature. Counterstain was applied using Mayer's hematoxylin, and sample dehydration was carried out by immersing the slides in 80%, 95%, and 100% alcohol; each was performed for 1 minute sequentially.

RESULTS
Population Characteristics and Research Locations

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slides are then re-evaluated to determine whether they can be read again; if there are slides that are damaged/unreadable, then the paraffin block is cut again. Baseline characteristics of the included population consist of age and sex. In this study, the mean age of the patients was 55.63 ± 12.7 years, with male sex as many as 19 patients (47.5%) and women as many as 21 patients (52.5%).
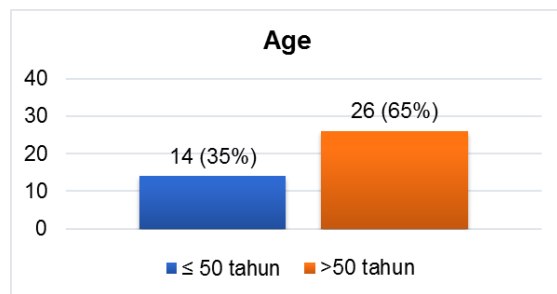

Figure 1. Age Group Proportions in Patiens with Colorectal Adenocarcinoma
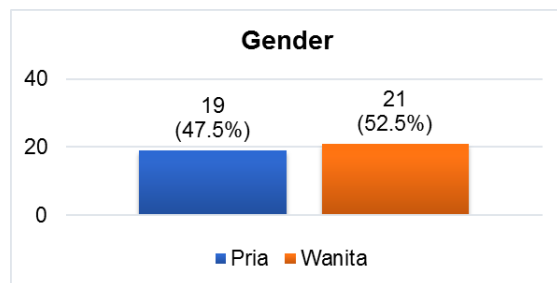

Figure 2. Gender Proportions in Patients with Colorectal Adenocarcinoma

Microscopic Observations – Degrees of Differentiation
Observations of the readings of the Il-6 expression will be categorized into intensity (weak = 1, strong = 2) and density calculated using ImageJ Fiji software (1 = 1%-50%; 2 = 51%-75%; > 76% = 3). The final score is obtained by multiplying the intensity result by the density (intensity score × density) and categorized as negative (score = 0), low

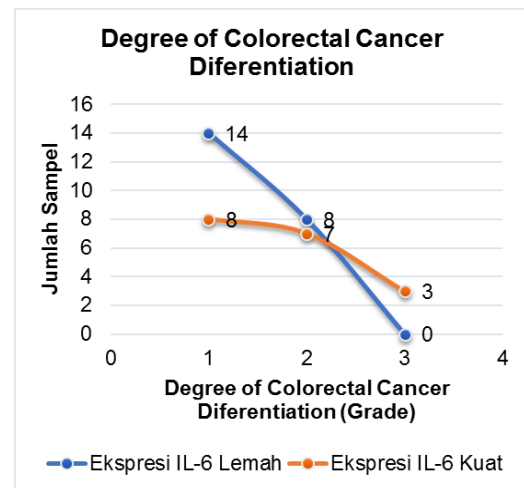expression (score $\leq$ 3), and high expression (score > 3).

Figure 3. Distribution of the Degree of Differentiation of IL-6 Expression in Colorectal Adenocarcinoma

Microscopic Examination – Degree of Infiltration
Microscopic examination is also used to assess the degree of tumor infiltration expressed by the letter 'T' / tumor. Microscopic examination related to the degree of tumor differentiation (graded from a score of 1 to 4), adenocarcinoma with differentiation degree 1 (one) totaling 22 samples (55%), grade 2 totaling 37.5%, and grade 3 totaling 3 (7.5%).

Spearman Correlation Statistical Test – Degree of Infiltration and Differentiation
The results of the statistical analysis aimed to find a correlation between the degree of differentiation (grade) of adenocarcinoma and IL-6 expression, as well as the degree of tumor infiltration (T) and IL-6 expression. (Table 5.1)

Table 1. Statistical Test Results

Variable	Analysis test	Significance; Correlation Coefficient	Comment
Gender with total IL-6 expression score	*Independent T-test*	0,01	Significance
Total IL-6 expression score with tumor grade	*Spearman*	0.039	Significance
Total IL-6 expression score with the degree of infiltration (T) of the tumor		0.129	Not significance

DISCUSSION
Characteristics of the Research Population

The final score of IL-6 expression in the sample was determined by multiplying the density and intensity of IL-6 expression. The scores obtained range from the smallest, namely 1 for 10 (25%) samples, score 2 for 9 (22.5%), score 3 for 3 (7.5%) samples, score 4 for 7 (17.5%) samples, and score 6 amounted to 11 (27.5%) samples.

Degree of Differentiation and Infiltration in Paraffin Block Samples

Based on microscopic observations, grade 1 = 22 samples (55%), grade 2 = 15 samples (37.5%), and grade 3 = 3 samples (7.5%); whereas for the degree of infiltration as assessed by the scoring system of the AJCC 8th edition, obtained as many as 1 (2.5%) samples with a degree of T1, T2 = 9 (22.5%) samples, T3 = 23 (57.5%), and degree of infiltration T4 = 7 (17.5%) sample.

Correlation Between Gender and Interleukin Expression – 6

This study's results align with the findings reported by Majek et al.,[15] that IL-6 expression was significantly higher in colorectal adenocarcinoma tissue of male patients compared to women. However, different results were reported by a study from India in 2017, that there were no significant differences in IL-6 expression in colorectal adenocarcinoma in both male and female patients.[16]

Differences that occur in the clinical outcome of patients with colorectal carcinoma between male and female sexes are influenced by various factors, one of the most important being hormones.[17] Based on data from the Global Cancer Observatory, the incidence of colorectal adenocarcinoma is 45% higher in males than females. However, in most cases, the incidence of colorectal carcinoma in the female population can equal that in the male population when the age reaches older people; namely 65 years or more; this phenomenon makes women experience a delay/delay from the incidence of colorectal carcinoma – with a frequency and possibility that is more frequent after stepping on the elderly period.[18]

There are various mechanisms of sex hormones influencing the proliferation and differentiation of malignant tissue. There is still much debate as to how sex hormones, which are known to be carcinogenic in large numbers of tissues, can exert a protective effect on the large intestine (sex hormone dimorphism) at the same time. The protective effect of estrogen on colorectal carcinogenesis causes a decrease in the inflammatory response through suppression of transcription and expression of NF-κB so that potent pro-inflammatory mediators are activated and release pro-inflammatory cytokines, such as TNFα, IL-6, and IL-1.[19]

Relationship Between IL-6 Expression and Tumor Differentiation

Statistical tests showed a significant correlation between the expression of interleukin-6 and the degree of tumor differentiation (p <0.05). Similar results were reported by Zeng et al., who stated that there was a significant relationship between

the degree of adenocarcinoma differentiation and IL-6 expression (p<0.05). [20] A similar study from Iran also stated the same thing: the level of IL-6 expression correlated with the degree of differentiation of colorectal adenocarcinoma.[21] The results of this study are also in line with a study conducted in Taiwan, with the results of a significant relationship between IL-6 expression levels based on microscopic examination with IHC staining and the degree of differentiation of rectal adenocarcinoma tumors..[22]

The different results reported in a study by Aditya et al., as assessed by a pathologist from microscopic examination, are unrelated to the degree of tumor differentiation. Differences in tissue IL-6 expression in several previous studies could be due to existing comorbidities, such as chronic hypertension, obesity, smoking habits, diabetes mellitus, and variations of existing genetic mutations. [23]

Relationship Between IL-6 Expression and Tumor Infiltration

The findings of this study indicate that there is no significant relationship between IL-6 expression and the degree of tumor infiltration. Another distinct finding is that IL-6 expression is significantly higher in tumors that have a deeper degree of invasion (T3/T4) than those that are more superficial.[24] Esfandi et al. investigated IL-6 expression in tissues by extracting colorectal tumor tissue and taking a supernatant sample from the tissue extract; the results showed that there was a significant correlation (p<0.001) between IL-6 expression and the degree of invasion of colorectal adenocarcinoma. [25]

IL-6 is a pleiotropic cytokine, namely a cytokine that can influence several phenotypic (multiple) properties and plays an important role in the process of growth and differentiation of cancer cells. Several studies have established the function of IL-6 on tumor growth and invasion, but the detailed mechanisms by which factors play the role are unclear; however, the effect of IL-6 on the growth and invasion of cancer cells depends on the type of cancer cell itself.[25] Another study in different organs by Tsui et al., who examined the degree of infiltration of the bladder, showed the same results as this study. In contrast, in the gallbladder tissue, there is a significant correlation between the degree of infiltration and the expression of interleukin-6. [26]

CONCLUSIONS

There is a significant correlation between the degree of differentiation and gender on the expression of interleukin-6 in colorectal cancer. In contrast, the degree of infiltration of colorectal cancer is not correlated to the expression of interleukin-6.

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