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Expression of Cd133 and Cd44 as Chemoresistance Prediction Factors in Unresectable Colorectal Carcinoma at Saiful Anwar Hospital

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Abstract
Backgroud
The presence of cancer stem cells (CSCs) in colorectal cancer (CRC) is correlated with disease progression and poor outcome. This study was aimed to investigate the correlation of CD44 and CD133 expression, which are a specific marker for CSCs, on the chemotherapeutic response in patients with unresectable CRC.
Methods
This study was designed as a cross-sectional study involving 38 subjects with unresectable CRC at Policlinic of Digestive Surgery, Saiful Anwar Hospital, Malang, Indonesia. Chemotherapeutic response were assessed by endoscopic evaluation post-chemotherapy. CD33 and CD144 expression were obtained by using immunohistochemistry method, while the calculation was done by using software ImageJ (ImmunoRatio and ImmunoMembrane plugins).
Results
Our data showed that majority of patients had high expression of CD44 (mostly 2+ or 3+) and CD133 (mostly 1+ or 2+) expression.Further analysis showed that CD44 expression and CD133 were negatively correlated with chemotherapeutic response ($p = 0.000$, $r = -0.555$; $p = 0.015$, $r = -0.391$; respectively). Logistic regression analysis showed that CD44 was more significantly affect the chemotherapeutic response compared with CD133.Chi-square analysis revealed that histopathology features (grading) and CEA level were associated with CD44 and CD144 expression.
Conclusion
There was a significant negative correlation between CD133 and CD44 expression with chemotherapy response in patient with unresectable CRC. CD44 expression was a stronger predictor for chemoresistance compared to CD133.

Keywords: *cancer stem cells, CD44, CD133, colorectal cancer*

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Based on American Cancer Society, colorectal carcinoma is the third of the most prevalent cancer and as the second cause of mortality in United States. In 2014, there were 96.830 and 40.000 new casesof colon and rectal cancer, respectively.[1] In agreement with that epidemiological data, data from Global Cancer Observatory issued by The International Agency for Research on Cancer (IARC) WHO reported that colorectal cancer was the third most prevalent cancer worldwide (approximately 10,2% or 1.8 million cases) and the second cause of cancer-related mortality (9,2% or 881.000 cases). In Indonesia, within 2018, the prevalence of CRC was 348.809 with approximately 8,6% or 30.023 were a new cases.[2-4]

Despite of a robust development of adjuvant treatment for CRC, the survival rate of patient with CRC still low, particularly in patient who firstly diagnosed as an advanced stage CRC. Data reported that more than 20% patients with CRC was diagnosed at stage 4. Furthermore, about 20-30% patient with CRC will be developed as metastatic disease during the course of treatment.[5]Interestingly, in spite of the development of personalized medicine, the incidence of resistance to treatment still high and this event lead to poor prognosis.

In general, tumor mass is composed from heterogeneous cell populations. Proliferation and differentiation assay on tumor cell population demonstrated that there are a specific sub-population within the tumor mass termed as Cancer Stem Cells (CSCs). CSCsare characterized by slow cell division, but have high

capability in self-renewal, unrestricted cell proliferation and multipotency, thus has a role in local recurrence, metastasis, and chemotherapy resistance.[6]The presence of CSCs has been found to be correlated with cancer progression, recurrence, metastasis, and resistance from conventional treatment.[7]Previous study reported that treatment targeting on the whole tumor mass yield partial regression that usually followed by the development of a new clonal arised from CSCs. Therefore, identification and targeting CSCs could potentially improve the strategy to specifically treating the cancer.Study reported that targeting CSCs cause stable tumor regression.[8]

Marker for CSCsin CRC are CD133, CD144, CD24, CD166, CD44, CD29, ALDH1, LGR5, and CXCR4.[9]CD133is expressed in the apical cells and these cells are involved in regulating cell to cell interaction, cell migration, and cell polarity. Previous study reported that CD133 cells could yield up to 2.5% tumorigenic cells. The presence of phenotype CD133 cells in CRCs could affect the tumor invasion (T) and regional lymph nodes expansion (N). CD133-positive cells are resistance from chemotherapy since it could attenuates the apoptotic effect of chemotherapeutic regimens.[10]

CD44 is a glycoprotein that involved in several biological processes such as cell growth, survival, differentiation, mobility, and cell interaction. This molecule is one of the main membranous receptor for hyaluronic acid.[11,12]Therefore, CD44 plays an important role in the remodeling and degradation of extracellular matrices components which provide a function on cell adhesion and migration related to tumor metastasis.[11,12] Study conducted by Huang and colleagues (2012) demonstrated that CD133 and CD44 co-expression was found in 40% of CRC patient.[13] Based on the importance of the role of CSCs in cancer development, recurrence, and chemotherapy resistance, this study was aimed to investigate the association of CD133 and CD44 expression with chemotherapy resistance in patient with unresectable CRC.

I. Methods

Study Design

This study was designed as retrospective cohort to investigate the association of CD133 and CD44 expression with chemotherapy resistance in patient with unresectable CRC in the Department of Surgery, Saiful Anwar Hospital, Malang, Indonesia. Categorization of unresectable colorectal cancer was defined as previously described in the literatures.[14,15]

Study Subjects

Subjects enrolled in this study was defined as a patient with established diagnosis of CRC who were managed as an outpatient care in Policlinic Digestive Surgery, Saiful Anwar Hospital and met the inclusion and exclusion criteria. Drop out criteria were defined as the subjects died during the study period or not attending the follow-up visit as being scheduled. Inclusion criteria were defined as follows: (1) Patient with CRC who categorized as unresectable CRC (evidence of metastasis [liver, lung, ovarium, non-regional lymph node, peritoneum], cT4 with high risk resection margin, including MRF or CRM + [usually was determined by MRI, eg., CRC case with lateral extension to lateral pelvic wall, sacrum, or other lateral compartement]), (2) age <70 years old, and (3) agree to participate this study (informed consent).

Exclusion criterias were defined as follows: (1) the presence of comorbidities such as the impairment in immune system, hepatic or renal dysfunction, metabolic disease, allergy or asthma, (2) pregnancy or breastfeeding. All subjects were obtained from CRC patient in Saiful Anwar Hospital who has been diagnosed for CRC, underwent biopsy, and determined to be managed by chemotherapy. Evaluation of immunohistochemistry staining for CD133 and CD44 from tumor tissue has been established as a requirement before starting the series of chemotherapy. Immunohistochemistry staining was done at Laboratory of Clinical Pathology, Saiful Anwar Hospital, Malang, Indonesia.

Clinical Procedure

After being provided by adequate information about the study, patients were enrolled in this study. At the time of admission, patients were thoroughly assessed for the general appearance, vital sign, and completion on staging. Clinical staging was done by thorough assessment on history, physical examination, and other diagnostic modalities (e.g., abdominal ultrasonography to proof the evidence of liver metastatic nodule, or MRI to find the lateral extension of tumor within pelvic compartements). Before starting the series of chemotherapy, biopsy was done to assess the histopathological features (grading) and the analysis for CD44 and CD144 expression. Carcinoembryonic antigen (CEA) levels were measured as the initial assessment of patient at Laboratory of Clinical Pathology, Saiful Anwar Hospital, Malang, Indonesia. Chemotherapy procedure was done using regiments 5 FU, leucoverin and avastin, every two weeks for six months. Response to chemotherapy was assessed by using endoscopic approach which was done at sixth months since the introduction of chemotherapeutic regiments. Categorization of chemotherapeutic response was defined as acomplete, partial, progressive, and stable response.Chemotherapeutic response based on RECIST 1.1 criteria, are as follows:

- Complete Response (CR): All target lesions disappeared over the course of the treatment (total target lesions = 0).
- Partial Response (PR): Decrease of total LD (longest diameter) size of tumor ≥ 30% of total target lesions.
- Progresive Disease (PD): Increase of total LD (longest diameter) size of tumor ≥ 20%, at least 5 mm of increment or the appearance of new lesion.
- Stable Disease (SD): Tumor size did not significantly decreased or increased on all target lesions.[16]

Measurement of CD133 and CD44 Expression

Tumor tissues were fixed in buffered formalin 10% for 18 hours. Large tumor tissues were firstly cut into smaller pieces (approximately 2-3 mm thickness). Fixed tissue then dehydrated ethanol, cleared in xylene, and the embedded in paraffin block. Embedded tissue then would be ready for sectioning (5 um thickness) by using microtome. Sectioned tissues were attached into poly-L-lysine-coated slides. Before staining process, attached tissues were incubated at 40˚C for 1 hour. After that, deparaffinization was done by using xylol I, II, and III for 3 minutes each. Sequentially, rehydration process was done by soaking the tissue into serial reduction ethanol (100%, 90%, and 80%). Following rehydration process, tissues were soaked in 0.5% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval procedure was done by soaking the sample in hot decloaking chamber. After this step, sample was incubated in room temperature for 30 minutes, rinsed with aquades, and then washed with PBS for 3 minutes. Subsequently, sample were put in moisture chamber and dropped with background sniper for 10 minutes.

Following antigen retrieval procedure, primary antibody of CD133 and CD44 were introduced and then incubated for 1 hour at room temperature or overnight at 4˚C. Before incubation with secondary antibody, samples were washed with PBS for 3 minutes. Secondary antibody were dropped on to tissues and incubated for 30 minutes. Sample then rewashed again with PBS for 3 minutes. Following this process, samples were incubated with Trekavidin-HRP Label for 40 minutes. Eventually, samples were dropped with DAB and incubated for 2-4 minutes (1 ml Betazoid Dab Substrate Buffer added with 1–2 drop of DAB Chromogen). After washed with running water for 5-7 minutes, samples were counter-stained with mayerhaematoxilinfor 2–3 minutes. Samples then soaked in saturated lithium carbonate for 3 minutes. After washing process using running water for 5-7 minutes, samples were dehydrated by using elevated concentration of ethanol (80%, 96%, 100%) and then cleared with xylol I, II, III for 3 minutes each. Samples then mounted using entellan and ready for imaging. Slide then scanned by light microscope (Olympus BX51) linked to camera and computer. CD144 and CD33 expression were calculated by using software ImageJ (plugin Immunoratio and ImmunoMembrane). CD 44 and CD 133 scoring in the Immunomembrane plugin (running inImage J) is based on both image intensity and "completeness" of staining around the circumference of tumor cells.These two independent scores are then summed, to createthe conventional 0 to 3+ score.

Statistical Analysis

Logistic regression was used to compare the correlation of CD133 and CD44 expression with chemotherapeutic response (compare the regression coefficient and Wald score to assess the significance of each variables). Chi-square test was performed to assess the association of CD133 and CD44 expression with gender, age, tumor location, histopathological features, and CEA levels.All of statistical procedures were done by using software SPSS for Windows version 22.0.

II. Results

Baseline Characteristics

There were 38 patients with unresectable CRCs involved in this study. Demographic data could be seen in Table 1. Majority of subjects were male subjects (60.53%) aged 40-60 years old (60.53%). Tumor location mostly at rectum region (63.16%) with initial CEA level more than 5 ng/mL (73.68%). Consistently, histopathological feature showed poorly differentiated or undifferentiated (39.47% and 26.32%, respectively). These data suggesting a high tumorigenic activity and extent invasion.

Table 1. Baseline Characteristics

Characteristics	Frequency	Percentage
Sex		
• Male	23	60.53
• Female	15	39.47
Age		
• < 40 years old	4	10.53
• 40-50 years old	10	26.32
• 50-60 years-old	13	34.21
• > 60 years old	11	28.95
Location		

• Colon	14	36.84
• Rectum	24	63.16
CEA Level (ng/mL)		
• < 5	10	26.32
• > 5	28	73.68
Histopathological Feature		
• Well-differentiated	4	10.53
• Moderately-differentiated	9	23.68
• Poorly-differentiated	15	39.47
• Undifferentiated	10	26.32
Chemotherapeutic Response		
• Partial response	1	2.63
• Complete response	1	2.63
• Progressive response	19	50.00
• Stable response	17	44.74
CD44 Expression		
• 0 or 1+	10	26.32
• 2+ or 3+	28	73.68
CD133 Expression		
• 0	7	18.42
• 1+ or 2+	31	81.58

Correlation between CD133 and CD44 Expression with Chemotherapeutic Response

Our data showed that majority of patients had high expression of CD44 (mostly 2+ or 3+) and CD133 (mostly 1+ or 2+) expression. This data suggesting that CD133 and CD44 play an important role in the CRC progression.Further analysis showed that CD44 expression was negatively correlated with chemotherapeutic response (Spearman correlation test, p = 0.000, r = -0.555). Consistently, CD133 was negatively correlated with chemotherapeutic response (Spearman correlation test, p = 0.015, r = -0.391). These data suggesting that both CD133 and CD44 expression could be used as a predictor for prognostic marker as they represent the chemoresistance possibility. Logistic regression test revealed that both CD44 and CD133 expression affect 64.9% (represented by Negelkerke R Square value) of the outcome of chemotherapeutic response. Further analysis revealed that CD44 was more significantly affect the chemotherapeutic response compared with CD133 (Table 2).

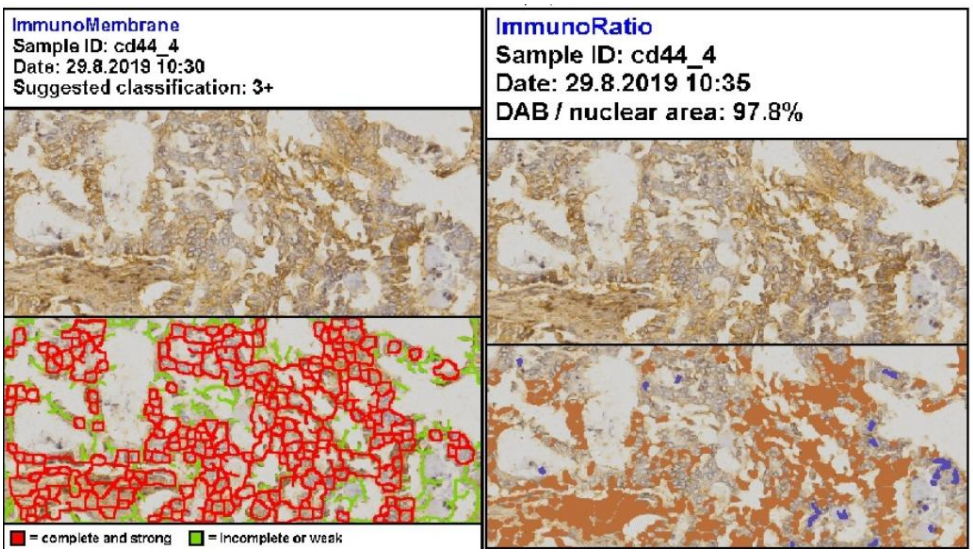

Figure 1. Immunohistochemistry staining on CD44 expression in primary colorectal carcinoma (400x magnification) and calculation process using ImmunoMembrane and ImmunoRatio plugins from ImageJ.

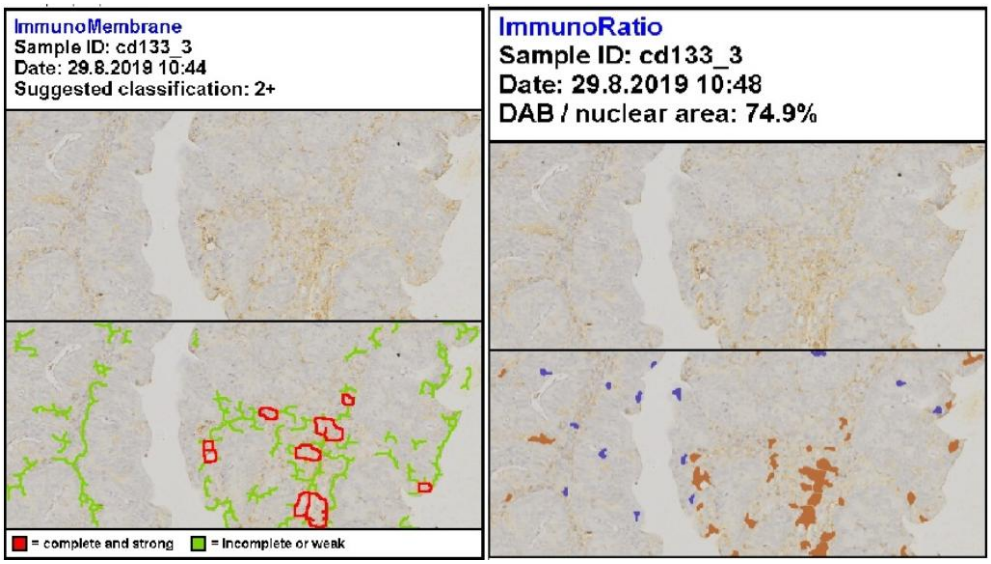

Figure 2. Immunohistochemistry staining on CD133 expression in primary colorectal carcinoma (400x magnification) and calculation process using ImmunoMembrane and ImmunoRatio plugins from ImageJ.

Table 2. Logistic regression test between CD133, CD44, and chemotherapeutic response

Independent Variables	B (regression coefficient)	Wald score	p-value
CD44	-2.986	6.197	0.013
CD133	-1.620	4.247	0.039

Correlation of Demographic and Clinical Parameters with CD133 and CD44 Expression

Analysis on factors associated with CD44 and CD133 expression showed that sex, age, and tumor location were not related to CD44 and CD133 expression (Pearson Chi-Square, $p > 0.05$). Interestingly, both CD44 and CD133 expression were related to histopathology features (grading) and CEA levels (Pearson Chi-Square, $p < 0.05$). Consistent with previous findings, these data suggested that both CD44 and CD133 expression were associated with elevated tumorigenic activity (proliferation and differentiation). Detail of association between CD44 and CD133 expression and each variables was shown in Table 3.

Table 3. Correlation of sex, age, tumor histopathology, location, and CEA level with CD44 and CD133 Expression

Variable	CD44		p-value	CD133		p-value
	0 or 1+	2+ or 3+		0	1+ or 2+	
Sex						
• Male	6	17	0.968	5	18	0.131
• Female	4	11		2	13	
Age (year)						
• ≤ 40	1	3	0.101	0	4	0.775
• 41-50	0	10		2	8	
• 51-60	6	7		3	10	
• ≥ 60	3	8		2	9	
Histopathology						
• Well-differentiated	3	1	0.012	3	1	0.023
• Moderately-differentiated	1	8		1	8	
• Poor-differentiated	6	9		2	13	
• Undifferentiated	0	10		1	9	
Location						
• Colon	3	11	0.601	4	10	0.218
• Rectum	7	17		3	21	
CEA Level (ng/mL)						
• ≤ 5	6	4	0.005	6	4	0.000
• > 5	4	24		1	27	

III. Discussion

Our data demonstrated that there was a significant correlation between CD44 and CD133 expression with chemotherapeutic response. Patient with moderate to high CD44 (2+ or 3+) or CD133 (1+ or 2+) has minimal response to chemotherapy (progressive and stable disease), thus correlated to poor prognosis. High expression of CD133 and CD44 are associated with high population of cancer stem cells.[17]

Recently, experimental study found that CSCs play an important role for tumor development as they have capability in self-renewal and considered to induce tumor growth. CSC has been found for its capability to endure the stress-induced chemotherapy, and this evidence could explain the phenomena of chemoresistance in our findings.[6] In agreement with this study, Morrison and colleagues (2008) reported that CSCswere the important risk factor for recurrence and mortality.[18] In fact, only a low number of tumor cell population were defined as CSCs which slowly growth but more resistant to chemotherapy and radiotherapy.[19]Chemoresistance properties of CSCs might be caused by cell adaptation process at cellular and genetic level (relatively dormant or slow cell-cycle kinetics, efficient DAN repair, expression of Transporter Multi Drug Resistance, and resistance to apoptosis.[7,20,21]

CD133 or previously known as prominin-1 is a transmembrane glycoprotein which present at cholesterol-containing lipid layer domain. Previous study reported that CD133 could activate DNA repair mechanism by strongly enhancing IL-4 production and inducing anti-apoptotic gene expression.[22]In clinical setting, high CD133 expression could potentially be used as a diagnostic or prognostic marker. As a prognosis predictor, CD133 was correlated with poor outcome, higher risk for metastasis, independent prognostic marker for overall survival, and associated with chemoresistance of 5-FU-based regiments or radiotherapy.[23] CD44 is a membranous glycoprotein which act as a receptor for hyaluronic acid and involved in cellular growth, survival, differentiation, mobility, and intercellular interaction.[22] Clinically, CD44 is associated with the depth of invasion, involvement of lymph nodes, distant metastasis, and consequently, reduced survival rate.[23]

Multivariate analysis showed that CD44 has a stronger association with chemotherapeutic response compared with CD133. This result was in accordance with the study conducted by Wang and colleagues (2012) which reported that the higher CD44 expression in colorectal cancer was associated with higher rate of tumor formation, tumor cell proliferation, reduction of spontaneous apoptosis events, and higher resistance to lethal factor.[24] CD44-positive cells has a stronger invasion capability compared with other cell phenotype.

Logistic regression analysis showed that gender, age, and initial CEA level did not affect the CD44 and CD133 expression significantly. Previous study demonstrated that there was no correlation between age and gender with the marker of CSCs in patient with colorectal cancer. Furthermore, same study also stated that age, tumor stage, vascular invasion, and CD133 expression were an independent prognostic factor.[25]Consistently, Jing and colleagues (2015) also reported that age, gender, and initial CEA level were not associated significantly with CD133 and CD44 expression.[26]

Management of cancer in elderly is challenging and more complex caused by several factors such as reduction of organ function, presence of co-morbidities, thus more susceptible to cytotoxic agents.[27] Moreover, age could influence the effectivity and safety of chemotherapeutic agents at three different levels (pharmacokinetics, pharmacodynamics, and normal tissue tolerance). An essential pharmacokinetics alterations are decreased glomerular filtration rate and volume distribution of water soluble agents.[28] Age and gender also associated with estrogen exposure which has protective role in colorectal cancer. Previous study reported that colorectal polyp or tumor was found more frequent in men.[29]The Women's Health Initiative showed that pre-menopausal women, which continuously exposed to estrogen, has 40% lower incidence of colorectal cancer compared with age-matched men. On the other hand, older women has worse overall survival rate compared to age-matched men. This phenomenon possibly associated with the reduction of estrogen hormone.

Signaling cascade of intercellular estrogen could affect both the reproductive and non-reproductive organs, including colon.[30]Estrogen receptor beta (ER-β) is a predominant estrogen receptors expressed in normal tissue or malignant epithelial of colon. During the development of colon cancer, ER-β is diminished, suggesting that estrogen signaling has a role in the progressivity of colon cancer. Estrogen mediates antitumor activity through selective activation of ER-β-mediated pro-apoptosis signal, inhibition of inflammatory signals, and modulation of microenvironment.[30]

Our data showed that there was no significant correlation between CD133 and CD44 expression with tumor histopathology and location. Conversely, study conducted by Kojima and colleagues reported that CD133 positive cells was found more frequent in well-differentiated tumor and this finding might be caused by differences of CD133 staining method (Hematoxylin-Eosin). Different antibody used could affect the staining pattern (cytoplasmic or membrane) and threshold value for positive cells.[24,31]Previous study conducted by Hong and colleagues reported that CD44 expression was associated with staging, poor differentiation, the depth of invasion, and the involvement of lymph nodes. Furthermore, Kojima and colleagues also reported that low expression of CD133 was found in tumor located in colon compared with rectum.[31]

IV. Conclusion

In conclusion, there was a significant negative correlation between CD133 and CD44 expression with chemotherapy response in patient with unresectable CRC. Furthermore, compared to CD133, CD44 expression was a stronger predictor for chemoresistance. Further analysis showed that there was a significant association between histopathological features and CEA level with CD133 and CD44 expression.

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