Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers, worldwide, with 1,360,600 clinically diagnosed new cases [1]. Epidemiology studies have revealed that approximately 25% of all patients who undergo colonoscopies are diagnosed with CRC [2]. The resistance and recurrence of CRC against most therapeutic options may be due to the existence of cancer stem cells (CSCs) in CRC tumors (CR-CSCs), which express multidrug resistance (MDR) pumps at high levels [3]. Although most standard cytotoxic therapies target rapidly dividing tumor cells, CSCs divide less frequently than other cancer cells, rendering them less susceptible to chemotherapeutic agents [4]. CSCs represent a small population of cancer cells that possess the characteristics of self-renewal and pluripotency and are responsible for the initiation and maintenance of tumors, including the development of metastatic tumors [5]. Various surface markers have been identified among the CR-CSCs population, particularly CD133 and CD166. Several factors have been associated with the activation of CSCs into tumor tissue, particularly tumor-associated macrophages (TAMs) [6].

TAMs are polarized type-2 macrophages that express a 130-kDa glycoprotein with an aminoterminal signaling element and a 9 scavenger receptor cysteine-rich (SRCR) domains [7]. Epidemiological studies have reported that TAMs, in most tumors, promote the development of aggressive tumors, with high metastatic potential, that are associated with poor prognoses [8]. TAMs are characterized by CD163 expression. We didn’t find any significant association of CD163 TAM with CRC histological grade and TNM stagings. Significant associations were found between the CD163 expression level and the CD166 expression level (p < 0.001). Increased TAM levels of CD163 was associated with 2.770-fold and 2.616-fold increased risks of elevated CD133 and CD166 levels, respectively.

CONCLUSION: An association was found between the expression levels of CD163 in TAMs and the expression levels of CD133 and CD166 in CR-CSCs.
the M2-macrophage-mediated release of inflammatory mediators [9]. Macrophages may become polarized into an 'M2-like' state, with several features of M2 cells [10]. The diversity of M2 macrophages can enhance the progression of tumors and metastasis [11].

The existence of TAMs in tumors may support the activation of CSC characteristics [12], [13]. A high degree of TAM infiltration in tumors has been associated with poorer prognoses in cancer patients [14]. The previous studies have reported that CRC enrichment can have a negative impact on CRC prognosis [15], [16] and may represent an independent predictor of survival among CRC patients [17]. Infiltrating TAMs are always distributed near CR-CSCs and the number of TAMs has been positively correlated with the histological grade of the malignancy and the number of CSCs. The existence of TAMs existence has been considered to be closely related to CSCs [18], [19]. However, whether association exist between the expression levels of TAMs genes and those in CR-CSCs remains unclear. The aim of this study was to investigate the association between the expression of CD163 in TAMs and the expression of proteins in CR-CSCs, including CD133 and CD166, in CRC patients.

Material and Methods

Patients and clinicopathological data

This study used a cross-sectional design that was approved by the Faculty of Medicine, Universitas Sumatera Utara, Adam Malik General Hospital Ethical Committee Board (533/TGL/KEPK FK USU-RSUP HAM/2018). We enrolled 118 patients, who were diagnosed with CRC, from September 2018 until July 2019. We collected the clinicopathological data, including gender, age, laboratory parameters, histological grading, and TNM stagings. The inclusion criteria for this study were primary colorectal adenocarcinoma and willingness to participate in the study, whereas the only exclusion criterion was a family history of CRC.

Immunohistochemical (IHC) staining method

The specimens were formalin-fixed and paraffin-embedded, sliced at a 4-µm thickness and stained with hematoxylin and eosin to histopathologically diagnose CRC. We utilized IHC staining for the examination of CD133, CD166, and CD163 marker expression in TAMs. To analyze the expression levels of these marker proteins, we used a primary anti-CD133 antibody, GTX83191 [10F1G12, 1:200–1:1000] (GeneTex International Corporation, California, USA), and a primary anti-CD163 antibody, GTX42365 [EDHu-1] (GeneTex International Corporation, California, USA). CD133, CD166, and CD163 expression levels were evaluated by two pathologists who did not have the patients' clinical information. An immunoreactivity score was calculated from the sum of both quantitative and qualitative parameters. A total score of 0–3 indicated low-level expression, whereas a total score of 4-6 indicated high-level expression.

For the quantitative analysis, the following scoring system, relative to the percentage of immunoreactive cells (% of the total area), was adopted, as previously described. Briefly, the percentages of immunoreactive cells were scored as follows: 0 (0% immunoreactive cells); 1 (<10% immunoreactive cells), 2 (10%–50% immunoreactive cells) and 3 (more than 50% immunoreactive cells). For the qualitative analysis, the immunoreactive staining intensity was classified according to the following scores: 0 (no immunoreactivity), 1 (weak immunoreactivity), 2 (intermediate immunoreactivity), and 3 (strong immunoreactivity). A combined score of <4, after the quantitative and qualitative analysis scores were added together, was considered to be "low-level" [20]. For the qualitative analysis of staining intensity, “strong” staining reflected intense staining similar to that observed for the positive control of the respective antibody. Figures 1 and 2 show high- and low-level CD 163 expression, respectively, as assessed by IHC staining in CRC samples. Figures 3 and 4 show high level of CD133 and CD166 expression respectively.

Figure 1: Immunohistochemical staining for CD163 in colorectal cancer samples. Low-level CD163 immunoreactivity appears as a light-brown color in the cytoplasm (red arrow). 400x magnification

Statistical analysis

Quantitative variables are presented as the mean ± standard deviation (SD) or as the median (minimum-maximum), for normal and abnormal distributions, respectively. The chi-squared test was...
used for comparisons between CD163 expression levels in TAMs and CD133 and CD166 expression levels in CR-CSCs. For statistical analyses, we considered p < 0.05 to be significant.

Figure 2: Immunohistochemical staining for CD163 in colorectal cancer samples. High-level CD163 immunoreactivity appears as a dark brown color in the cytoplasm (red arrow), 400x magnification

Results

The mean age of included patients was 57.17 ± 12.99 years old. Of the 118 total CRC patients, 69 (58.5%) were males and 49 (41.5%) were females. CD163 expression levels are shown in Figures 1 and 2. The characteristics of all subjects are presented in Table 1. No significant differences were observed between high-level and low-level CD163 expression, based on gender, age, or laboratory parameters, as shown in Table 2.

Table 1: Baseline characteristics of subjects

| Variable          | n (%)       |
|-------------------|-------------|
| Gender            |             |
| Male              | 69 (58.5%)  |
| Female            | 49 (41.5%)  |
| Age (years)       | 57.30 ± 12.99 |
| CD163 expression  |             |
| High-Level        | 45 (38.1%)  |
| Low-Level         | 73 (61.9%)  |
| CD133 expression  |             |
| High-Level        | 44 (37.3%)  |
| Low-Level         | 74 (62.7%)  |
| CD166 expression  |             |
| High-Level        | 43 (36.4%)  |
| Low-Level         | 75 (63.6%)  |

The comparisons between CD163 expression levels and those for CD133 and CD166 are shown in Tables 3 and 4, respectively. High-level CD163 expression in TAMs increased was associated with a 2.770-fold increase in the risk of high-level CD133 expression and with a 2.616-fold increase in the risk of high-level CD166 expression. There are no significant association between CD163 TAM and CRC histological grading nor TNM staging (Tables 5 and 6).

Table 3: Comparisons between CD133 and CD163 expression levels

| Variable  | CD133 | CD163 | p-value | PR (95% CI) |
|-----------|-------|-------|---------|-------------|
| Age (years) |       |       |         |             |
| High-level | 27 (62.8%) | 16 (37.2%) | <0.001  | 2.770 (1.719–4.464) |
| Low-level  | 17 (22.7%) | 58 (77.3%) |           |             |

Table 4: Comparisons between CD166 and CD163 expression levels

| Variable  | CD166 | CD163 | p-value | PR (95% CI) |
|-----------|-------|-------|---------|-------------|
| Age (years) |       |       |         |             |
| High-level | 27 (62.8%) | 16 (37.3%) | <0.001  | 2.616 (1.645–4.160) |
| Low-level  | 18 (24%) | 57 (76%) |           |             |

Discussion

TAMs play an important role in the regulation of the tumor microenvironment and the maintenance of the CSC niche [21]. TAM scan increase tumor growth by supporting angiogenesis, tumor progression, invasion, and metastasis [22]. CR-CSCs represent a small population of CR cells that are characterized by the expression of CD133 and CD166. CD133, also known as prominin-1 is a five-transmembrane glycoprotein primarily localized in membrane protrusions [23], which impacts the development of radiochemotherapy resistance in CRCs [24]. CD166 is an important CSC marker, functionally involved in cell-cell interactions, T-cell proliferation, hematopoiesis, and angiogenesis [24], [25]. CD163 is a characteristic TAM protein that acts as the scavenger receptor for the hemoglobin (Hb)-haptoglobin complex [26]. In this study, we investigated the association between CD163 expression in TAMs and the expression of several CSC markers that have previously been reported to impact CRC prognosis, including CD133 and CD166.
CR-CSCs and TAMs, during which CR-CSCs release chemoattractant molecules, such as chemokine ligand 2 (CCL2), CCL5, and vascular endothelial growth factor (VEGF)-A, to promote the infiltration of macrophages and encourage their polarization into an M2 phenotype [28]. In contrast, TAMs express growth factors that activate CSCs, leading to tumor formation and the development of antitumor drug resistance [29]. Furthermore, these interactions may stimulate the secretion of platelet-derived growth factor (PDGF)-BB, inducing the activation of stromal cells, which secrete fibroblast growth factor (FGF)7 and FGF9 for CSC proliferation [30].

Table 5: CD 163 expression levels based on histological grade

| Histological grade   | CD 163 TAM expressions | Total | p     |
|----------------------|------------------------|-------|-------|
|                      | High level             | Low level |       |
| Well differentiated   | 16 (29.1%)             | 39 (70.9%) | 55    | 0.291 |
| Moderately differentiated | 21 (43.8%)       | 27 (56.2%) | 48    |
| Poorly differentiated | 6 (40%)                | 9 (60%)  | 15    |
| Total                | 43 (36%)               | 75 (63%)  | 118   |

TAMs also express milk-fat globule epidermal growth factor VIII (MFG-E8), which is involved in angiogenesis, phagocytosis, and immune tolerance. MFG-E8 induces CSCs to form tumors and promotes antitumor drug resistance via the signal transducer and activator of transcription (STAT)3 and hedgehog signaling pathways [31]. The absolute number of macrophages and the balance between activating and suppressing macrophages can influence tumor behavior. A low number of intra tumoral type 2 and a high number of activating type 1 macrophages have been correlated with reduced cancer recurrence and liver metastasis, which can be used to predict cancer prognosis [32].

In this study, we also found no differences in Hb levels between patients with high- and low-level CD163 expression. We did not classify decreases in Hb levels based on anemia grades or the time span of anemia. Severe, chronic anemia can lead to hypoxic tissues, which may induce the activation of CSCs, and TAM scan also be affected by hypoxia-related factors. Hypoxia may decrease TAM mobility and increase the number of TAMs found in tumors [33]. TAMs are involved in DNA damage and cancer-related inflammation, through the release of inflammatory mediators, such as IL-6 and tumor necrosis factor (TNF)α [34]. The release of cytokines, such as IL-6 and TNF-α, by TAMs may affect tumor cell invasion and stromal cells [35], [36], [37].

Table 6: CD 163 expression levels based on TNM stagings

| TNM staging | CD 163 TAM expressions | Total | p     |
|-------------|------------------------|-------|-------|
|             | High level             | Low level |       |
| Stage 1     | 13 (25.5%)             | 38 (74.5%) | 0.079*|
| Stage 2     | 6 (42.9%)              | 8 (57.1%) |       |
| Stage 3     | 17 (40.5%)             | 23 (59.5%) |       |
| Stage 4     | 7 (63.6%)              | 4 (36.4%) |       |
| Total       | 43 (36.4)              | 75 (63.6%) | 118   |

*Fisher’s exact test.

One limitation of this study was that we did not assess the levels of other inflammatory factors, such as IL-6, MFG-E8, IL-11, transforming growth factor-β, or cells, such as T-helper 2 or regulatory T cells, which may explain the mechanism through which CD163 expression in TAMs can influence protein expression in CS-CRCs. Further studies should explore this mechanism, especially in CRC.

Tumor histological grade might be considered to be correlated with TAMs functions. A previous study reported that the more malignant the histopathology fenotype associated with macrophage infiltration and extensive stromal reactions in CRC [38]. There was significant association of TAMs density and histological grade [39], [40], [41]. In this study, we didn’t find significant association of CD163 TAMs and CRC histological grade (p = 0.291). The difference results might be due to the marker that used for TAMs were different such as CD 68, meanwhile in this study we used CD 163 as TAMs marker. In this study, we also didn’t find any significant association of CD 163 TAM and TNM stagings. This finding was similar to the previous studies that didn’t find association among these [40], [42], [43].

One limitation of this study was that we did not assess the levels of other inflammatory factors, such as IL-6, MFG-E8, IL-11, transforming growth factor-β, or cells, such as T-helper 2 or regulatory T cells, which may explain the mechanism through which CD163 expression in TAMs can influence protein expression in CS-CRCs. Further studies should explore this mechanism, especially in CRC.
Conclusion

We found an association between CD163 expression levels in TAMS and the expression of the CSC markers CD133 and CD166.

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